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BLOOD- AND MILK-LEVELS PRODUCED BY SULPHONE\*  
AND VARIOUS SULPHONAMIDES IN DOMESTIC  
ANIMALS

By

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## INTRODUCTION

THE therapeutic efficiency of the sulphonamides depends on the tolerated blood-levels which they produce and their antibacterial potency. Their relative potencies against two important animal pathogens, namely, *Str. agalactiae* and *Past. septica*, were determined by Francis, Peters and Davies (1947a, b) and Francis (1946a, 1947a). The work of Stableforth and Hignett (1942) provides accurate data on the behaviour of sulphanilamide in the domestic animals; but when the work described here was undertaken information on the other sulphonamides was scattered and incomplete. It was reviewed by Francis (1946b), who also determined the blood-levels produced by repeated doses of the sulphonamides in various domestic animals and discussed the application of the findings to the treatment of animal diseases (Francis, 1947a, b).

In this paper the blood-levels produced by single oral, intravenous or intraperitoneal doses of the sulphonamides are described. It was shown by Rose and Spinks (1946) that there is a considerable variation in the blood-levels produced by the same drug in different individuals and they used at least ten groups of three mice to get accurate data. It would clearly be a very large undertaking to compare the behaviour of eight drugs in such large groups of cattle, horses, sheep and pigs and three animals of each species have generally been used. With some of the drugs there was considerable variation in the blood-levels produced in different individuals and in these instances not all points fall on the curves shown in the graphs; the best average curves have been drawn, however, and the findings reported in this paper have been broadly confirmed by the administration of repeated daily doses of the sulphonamides to the various species (Francis, 1947b).

## EXPERIMENTAL

Except where otherwise stated, all animals were dosed orally at the rate of 0.1 gm./kgm. and intravenously or intraperitoneally at a dose of 0.05 gm./kgm. Soluble sodium salts of all the drugs we wished to inject intravenously could not be prepared and, as it has been shown by Francis (1947b) that soluble organic derivatives are not excreted at the same rate as their parent compounds, all drugs were administered as suspensions; no ill effects were observed.

\* Except where otherwise stated, sulphone refers to 4:4' diaminodiphenyl sulphone.

The concentrations of the sulphonamides in the blood were determined by the method of Rose and Bevan (1944).

### Pigs

As oral dosing of pigs is not easy, the drugs were fed to groups of three pigs in a small amount of mash. Samples were taken after intervals of 1, 2, 3, 5, 7, 24 and 48 hours. Absorption was rapid and the results were probably more regular than in calves or sheep. All the results were obtained with six pigs which were divided into two groups. One drug, "Sulphamezathine," was fed to both groups and very similar blood-levels were obtained in each. The average results are shown in Fig 1. In the pig "Sulphamezathine" was the most persistent drug, whereas in all the other domestic animals sulphone produced the best blood-levels.

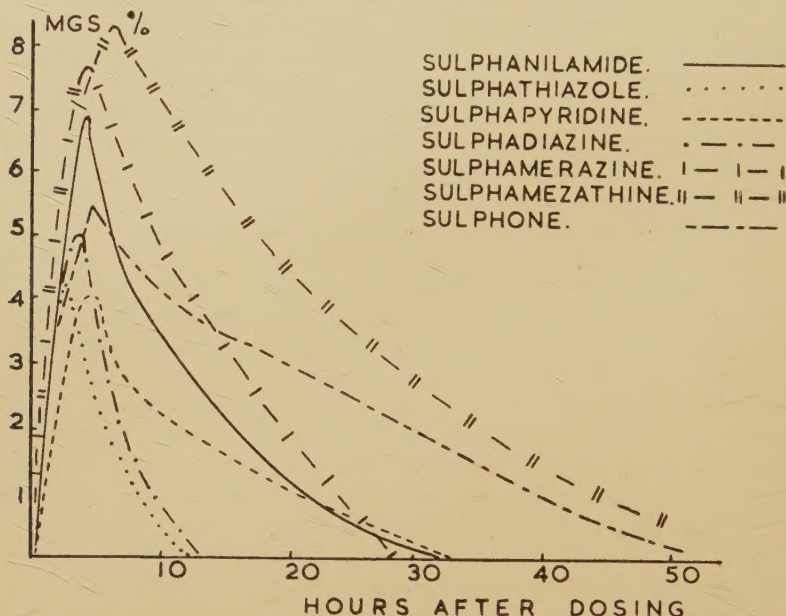


FIG. 1.—Average blood-levels produced in groups of three pigs by the oral administration of 0.1 gm./kgm.

*Footnote:* During the course of these experiments, which occupied several weeks, the weights of the pigs varied from 20 to 40 kgm.

Pigs were not injected intravenously because this is often impossible with the large bore needles which have to be used for suspensions. Instead they were inoculated intraperitoneally and the results resembled those following oral dosing rather than intravenous injection. They are not shown in detail, but are summarised in Table IV. The levels for sulphamerazine were surprisingly low, but the experiments were repeated on a group of pigs which had given good blood-levels with "Sulphamezathine" and similar results were obtained.

## Horses

The dose of each drug was mixed with a small feed, but the horses did not readily eat their food when it contained sulphanilamide, sulphathiazole or sulphapyridine. These drugs were therefore suspended in water and administered orally after the horses had eaten. Six horses were used in these experiments and they were usually divided into the same two groups. "Sulphamezathine" was fed to both groups and the blood-levels produced in each were very similar. Samples were taken after intervals of 1, 2, 3, 5, 7 and 24 hours and the results are shown in Fig. 2. The percentage of methaemoglobin produced by sulphone was determined by Dr. A. Spinks using the method of Vandebelt *et al.* (1944). The method

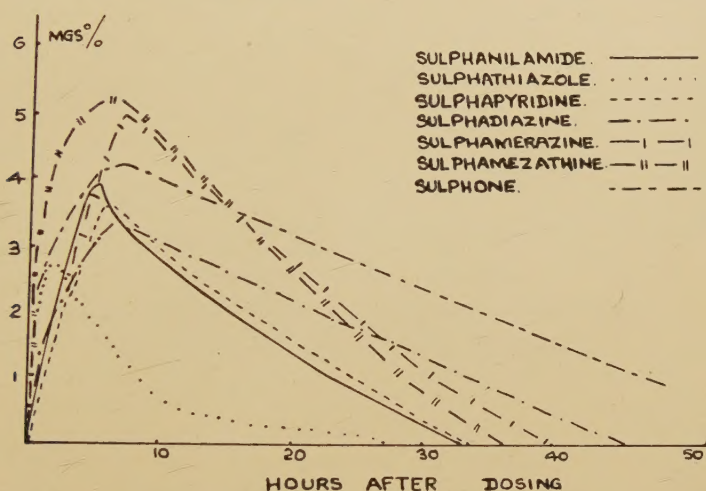


FIG. 2.—Average blood-levels produced in groups of three horses by the oral administration of 0.1 gm./kgm.

Footnote: The weights of the horses varied from 460 to 520 kgm.

differentiates between methaemoglobin and sulphaemoglobin and it seems improbable that any other pigment likely to be present would give the same absorption curve. Two to three hours after dosing nearly 70 per cent. of haemoglobin was in the form of methaemoglobin, but the level fell sharply to below 40 per cent. by the time the maximum level of sulphone had been reached eight hours after dosing. This finding is rather surprising, but on two occasions a similar phenomenon occurred in sheep. In this species sulphone may produce toxic nervous symptoms which reach a maximum and then decline, whilst the blood-level of sulphone is still rising.\*

\* These results are of interest in view of the now well recognised fact that patients develop a tolerance to sulphone or its derivatives.

*Sheep*

Sheep were dosed individually from a bottle and blood samples were taken at hourly intervals for the first seven hours and then morning and evening until they became negative. The results following oral administration are shown in Fig. 3 and following intravenous inoculation in Figs. 4 and 5. Following intravenous

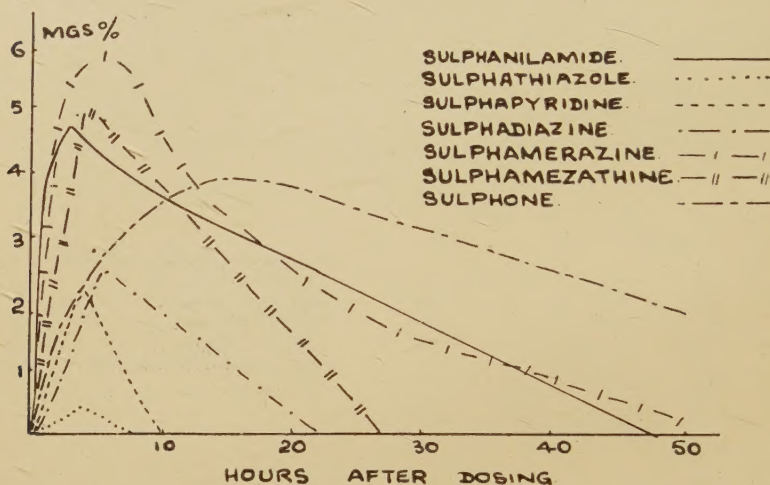


FIG. 3.—Average blood-levels produced in groups of three sheep by the oral administration of 0.1 gm./kgm.

Footnote: The weights of the sheep varied from 45 to 70 kgm.

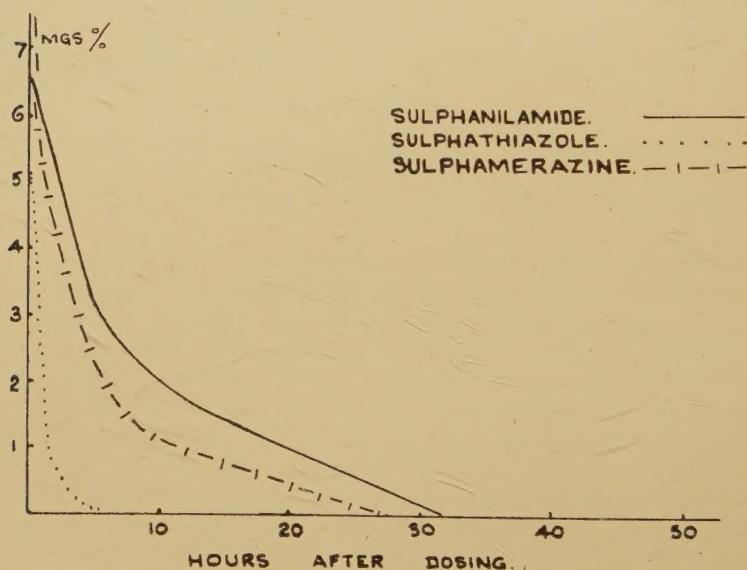


FIG. 4.—Average blood-levels produced in groups of three sheep by the intravenous administration of 0.05 gm./kgm.

Footnote: The weights of the sheep varied from 40 to 70 kgm.

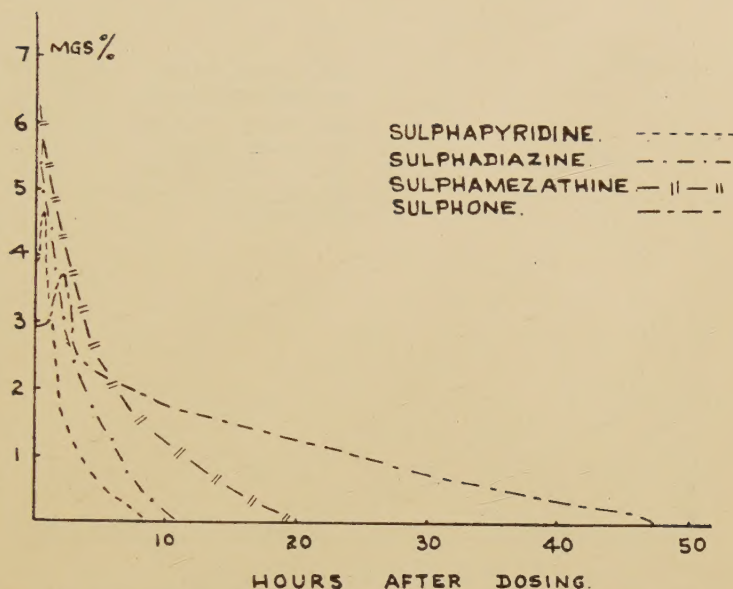


FIG. 5.—Average blood-levels produced in groups of three sheep by the intravenous administration of 0.05 gm./kgm.

Footnote: The weights of the sheep varied from 40 to 79 kgm.

injection samples were taken after intervals of 20, 40, 60 and 90 minutes and then continued as with oral dosing. It is of interest that the blood-level of sulphone was higher two hours after intravenous injection than it was at earlier intervals. It seems probable that this was because particles of the drug were arrested in the lung and took some time to dissolve. The same phenomenon was observed in the calf. Sheep were also dosed orally at a rate of 0.2 gm./kgm. The results are not shown in detail but are summarised in Table III. One sheep given this dose of sulphone died after 56 hours, when it had a blood-level of 6.5 mgms. per cent. Sheep were also given oral doses of 0.05 to 0.4 gm./kgm. of 4'nitro: 4'aminodiphenyl sulphone and the blood-levels produced indicated that it behaved in a similar manner to 4:4' diaminodiphenyl sulphone.

### Calves

Calves were dosed individually from a bottle. Samples were taken at the same intervals as with sheep and the results are shown in Fig. 6. Sulphone was not toxic to calves even when given in doses of 0.4 gm./kgm. Calves were also dosed orally with 4'nitro: 4'amino-diphenyl sulphone and the results indicated that the drug behaved in a similar manner to 4:4'diaminodiphenyl sulphone.

The results following intravenous administration of the various drugs are shown in Figs. 7 and 8.

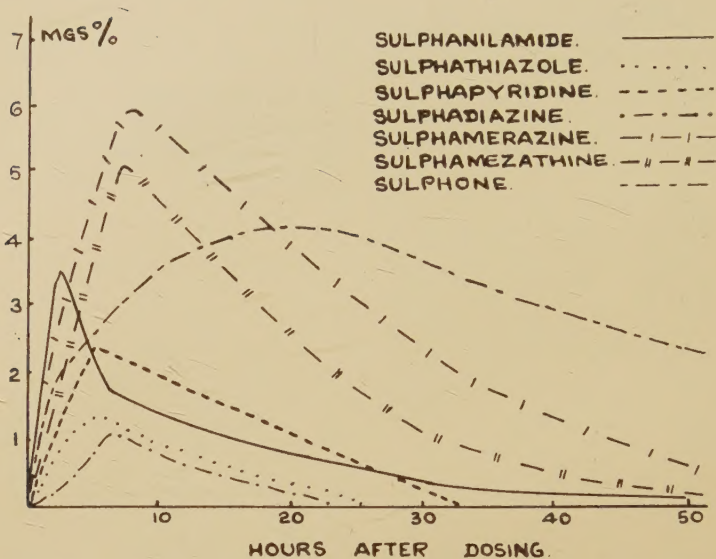


FIG. 6.—Average blood-levels produced in groups of three calves by the oral administration of 0.1 gm./kgm.

Footnote: The weights of the calves varied from 34 to 111.5 kgm.

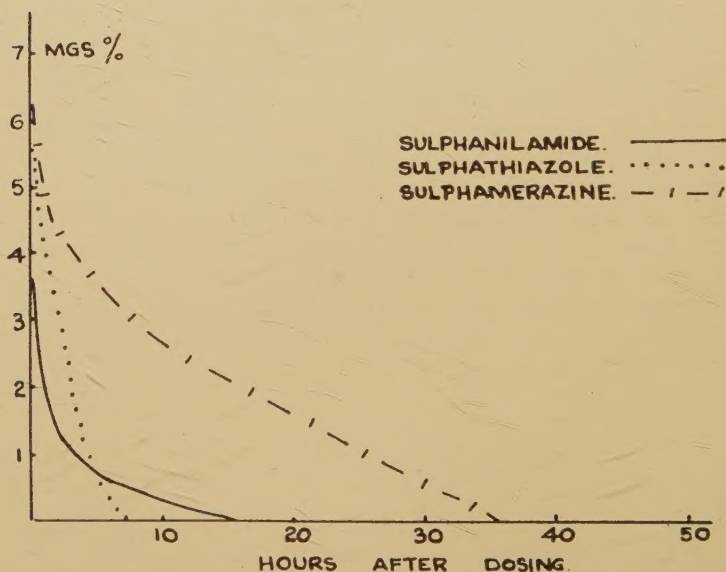


FIG. 7.—Average blood-levels produced in group of three calves by the intravenous administration of 0.05 gm./kgm.

Footnote: The average weights of the groups varied from 39 to 44 kgm.

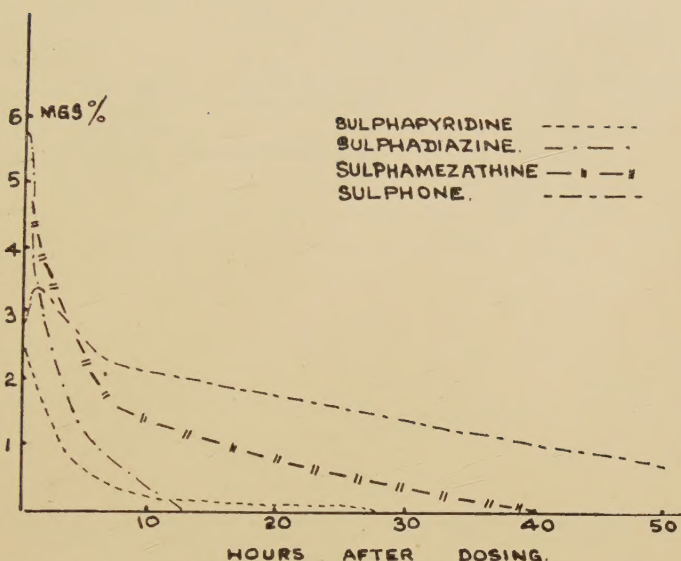


FIG. 8.—Average blood-levels produced in groups of three calves by the intravenous administration of 0.05 gm./kgm.

Footnote: The average weights of the groups varied from 39 to 44 kgm.

### Cows

The chief use of the sulphonamides in the cow is for the treatment of mastitis. The evidence indicated (Francis, 1947a) that only sulphone would be likely to replace sulphanilamide for this purpose, so these two drugs alone were studied. Three cows were dosed individually from a bottle with sulphanilamide and one week later with sulphone. Samples were taken at the usual intervals and the blood- and milk-levels obtained are shown in Fig. 9. It will be seen that, although sulphone produced higher blood-levels than sulphanilamide, it produced lower milk-levels.

A few observations were made on the acetylation of the sulphonamides. The degree of acetylation was determined in six pairs of calves which had been dosed with 0.1 gm./kgm., followed by half this dose of the various drugs on four successive days. The average results of each pair of calves are shown in Table I. Sulphathiazole, sulphanilamide and sulphapyridine were highly acetylated. "Sulphamezathine" was the least acetylated and sulphadiazine occupied an intermediate position. Acetylation is one obvious method by which the free drug disappears from the blood and the low acetylation of "Sulphamezathine" is clearly an important factor in accounting for the good levels which it produced in the calf. Sulphone was quite highly acetylated, but there were good levels of the free drug and the total level was nearly twice as high as that for any other drug.

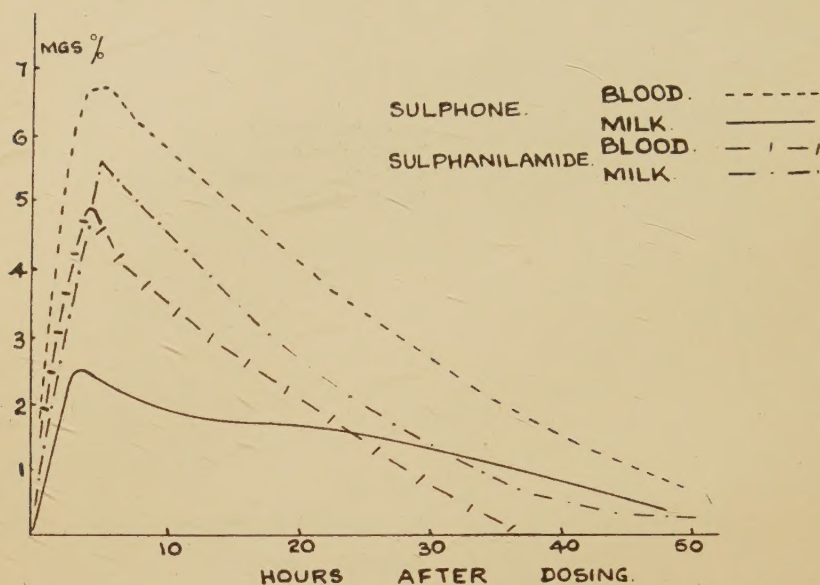


FIG. 9.—Average blood- and milk-levels produced in a group of three cows by the oral administration of 0.1 gm./kgm.

It was thought that the poor blood-levels produced by sulphanilamide in the calf might be due to excessive acetylation. A dose of 0.1 gm./kgm. of sulphanilamide was given to two cows and two calves and one week later the same animals were given a dose of 0.1 gm./kgm. of "Sulphamezathine." Blood samples were taken at intervals of 1, 2, 4, 5, and 24 hours and the total and free sulphonamide levels determined. The average level of free sulphanilamide in the cow was 4.04 mgm. per cent. and 41.3 per cent. of the total

TABLE I

THE DEGREE OF ACETYLATION AFTER THE ORAL ADMINISTRATION OF 0.1 GM./KGM., FOLLOWED BY HALF THIS DOSE FOR FOUR MORE DAYS, OF SULPHONE AND VARIOUS SULPHONAMIDES TO CALVES \*

| Calf Nos. | Drug            | Level of "Total" drug | Level of "Free" drug | Percentage of "Total" drug acetylated |
|-----------|-----------------|-----------------------|----------------------|---------------------------------------|
|           |                 | mgm. %                | mgm. %               |                                       |
| 52 and 55 | Sulphanilamide  | 3.7                   | 1.1                  | 70.2                                  |
| 51 „ 56   | Sulphathiazole  | 3.0                   | 0.5                  | 83.3                                  |
| 55 „ 54   | Sulphapyridine  | 3.7                   | 1.25                 | 66.2                                  |
| 56 „ 53   | Sulphadiazine   | 4.3                   | 2.7                  | 37.2                                  |
| 54 „ 52   | Sulphamezathine | 4.8                   | 4.2                  | 12.5                                  |
| 53 „ 51   | Sulphone        | 8.8                   | 5.5                  | 37.5                                  |

\* More details concerning these calves are given by Francis (1947b). The results are based on a single estimation carried out on the fourth day.

was acetylated. In the calves the average level of the free drug was 2.1 mgms. per cent. and 54.7 per cent. of the total drug was acetylated. With "Sulphamezathine" the degree of acetylation in the cow and calf was about 20 per cent., but better levels of the free drug were produced in the cow than the calf, a result which confirms those in Table I. Taken together, the results did not indicate that the big differences in the levels of free sulphanilamide in the cow and calf were due to excessive acetylation in the calf, but they confirmed that "Sulphamezathine" was much less acetylated than sulphanilamide in the calf.

### Mice

Ten groups of three mice were dosed orally with 0.05 gm./kgm. of sulphanilamide and sulphone respectively. The blood-levels were determined on pooled samples from each group of three mice and the average results are shown in Table II.

TABLE II

AVERAGE BLOOD-LEVELS PRODUCED BY THE ORAL ADMINISTRATION OF 0.05 GM./KGM., (1 MGM./20 GM.) OF SULPHANILAMIDE AND SULPHONE TO TEN GROUPS OF THREE MICE

| Drug                               |     | Time in hours |      |      |      |      |      |      |      |      |
|------------------------------------|-----|---------------|------|------|------|------|------|------|------|------|
|                                    |     | 1/3           | 2/3  | 1    | 1½   | 2½   | 3½   | 5    | 7    | 24   |
| Sulphanilamide                     | ... | 2.03          | 2.65 | 2.7  | 1.85 | 1.3  | 1.19 | 0.74 | 0.35 | 0.04 |
| 4 : 4' diaminodiphenyl<br>sulphone | ... | 1.51          | 1.95 | 2.36 | 2.43 | 2.11 | 1.82 | 1.56 | 1.0  | 0.2  |
| Average                            | ... | 1.77          | 2.30 | 2.53 | 2.14 | 1.70 | 1.50 | 1.15 | 0.67 | 0.12 |

### Other species

As already stated when the work described in this paper was begun there was little information on the behaviour of the newer sulphonamides in the domestic animals. There is now more precise comparative data in animals than in man, and although all investigators have not used the same dose evidence is available which makes it possible to apply corrections. It will be seen that increasing the oral dose in the sheep from 0.1 to 0.2 gm./kgm. increased the blood-level curve area (B.L.C.A.)\* threefold (Table III). Rose and Spinks (1946) gave mice oral doses of 0.25 gm./kgm., and it is interesting that the ratio of the B.L.C.A.s produced by this dose of sulphanilamide and the dose of 0.05 gm./kgm. used in the present study was 7.2:1 and not 5:1. This provides further evidence that doubling the dose of a sulphonamide more than doubles the B.L.C.A. If the results of

\* The large number of results obtained in the foregoing studies cannot easily be compared unless the behaviour of each drug in each species can be given a simple numerical expression. This can be done by measuring the area enclosed by the respective graphs. This "blood-level curve area" (B.L.C.A.) is an expression of the level obtained and the time for which it persists.

Stableforth and Hignett (1942) in the cow, horse and dog are averaged and graphed it can again be shown that doubling the dose of sulphanilamide over the approximate range of 0.1 to 0.2 gm./kgm. produced a threefold increase of the B.L.C.A. As already mentioned, Rose and Spinks (1946) used an oral dose of 0.25 gm./kgm. in the mouse. To compare their results with ours we have divided the B.L.C.A.s by three, which from the foregoing evidence is certainly not too large a figure.

The figures for the cow are based largely on the results of Maclay and Slavin (1947), who gave oral doses of 0.15 gm./kgm. The B.L.C.A.s obtained for these figures were therefore divided by 2.2 and the results are shown in Table III, together with those obtained with sulphanilamide and 4:4'diaminodiphenyl sulphone in this study (Fig. 9). Marshall (1945) gave chicks weighing 50 to 100 gm.

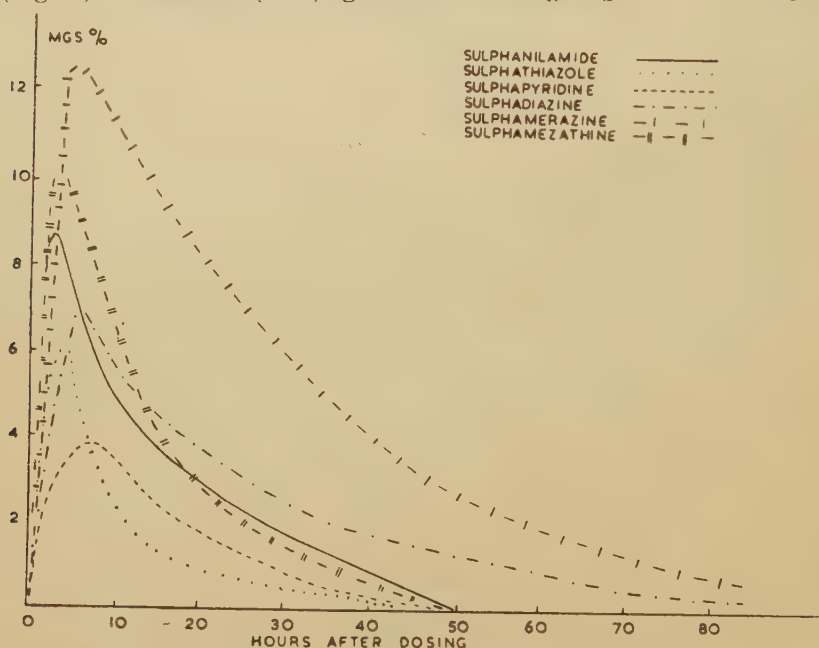


FIG. 10.—Average blood-levels produced by the oral administration of 4 gm. (approx. 0.06 gm./kgm.) of various sulphonamides to man.

*Footnote:* The data on which these curves were based was collected from the literature by Dr. A. Spinks. Some doses slightly above and some slightly below 4 gm. have been included. The references on which the data for each drug is based are shown below:

*Sulphanilamide:* Frisk (1943).

*Sulphapyridine:* Barlow and Climenko (1941); Long and Feinstone (1938); Frisk (1943).

*Sulphathiazole:* Barlow and Climenko (1941); Frisk (1943); Long *et al.* (1940).

*Sulphadiazine:* Peterson *et al.* (1941); Welch *et al.* (1943); Goodwin *et al.* (1942); Frisk (1943).

*Sulphamerazine:* Welch *et al.* (1943); Murphy *et al.* (1943); Goodwin *et al.* (1942).

*Sulphamezathine:* Volini *et al.* (1945); Welch *et al.* (1943); Rose *et al.* (1943).

The data for rabbits and chicks is incomplete.

oral doses of 0.25 gm./kgm. As in the mouse, we have divided the B.L.C.A.s obtained after redrawing his curves as accurately as possible by three.

The levels for the dog and cat are taken from the paper of Welsh *et al.* (1946), who used a dose of 0.07 gm./kgm. As a comparison of their results in the pig, horse and sheep showed that they obtained almost the same levels as in this study, in which a dose of 0.1 gm./kgm. was used, their levels for the dog, cat and rabbit have been accepted as comparable with our own. Welsh *et al.* (1948) gave foxes doses of 0.07 gm./kgm. and their results have been treated in the same way. The figures for man were collected from the literature by Dr. A. Spinks. They represent the levels produced by doses of 4 gm. to a man or approximately 0.06 gm./kgm. They are shown graphically in Fig. 10 because this is believed to be the first time that a complete presentation has been made.

#### DISCUSSION\*

*Behaviour of the Sulphonamides as a Group.*—It will be seen from Table III that the average B.L.C.A. produced by the sulphonamides in the pig, horse, cow and cat are considerably greater than in the mouse. The average B.L.C.A. in the fox is slightly less than in the mouse, and the dog, calf and sheep occupy an intermediate position. The curves in Fig. 11 show that the pig absorbed the drugs considerably, and the horse slightly, more rapidly than the calf or sheep, in which animals the average curves were very similar. Complete data are not available for the rabbit, but the

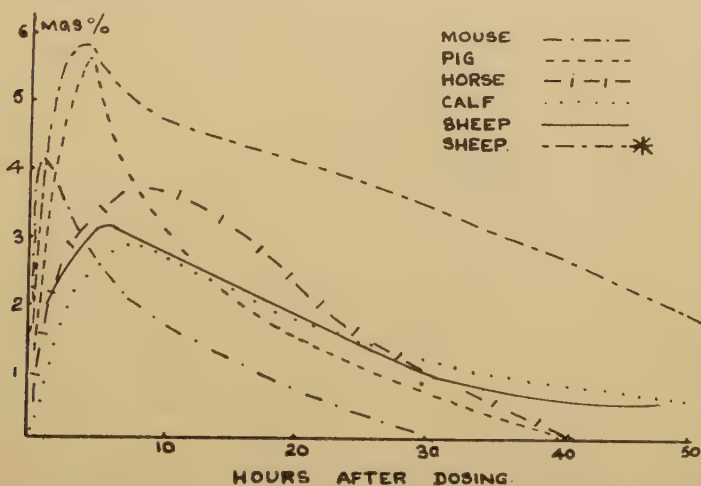


FIG. 11.—Average blood-levels produced by the oral administration of 0.1 gm./kgm. of sulphone and the various sulphonamides in five animal species.

Footnote: Sheep were also dosed orally with 0.2 gm./kgm. of the same drugs.

\* For a general discussion on the sulphonamides, see Hawking (1945).

sulphonamides obviously produce very low levels in this animal which is largely due to excessive acetylation (Corwin, 1941; Alexander, 1943). Again, complete data are not available for the chick, but it is obvious that the sulphonamides produce considerably lower levels than in the mouse, despite the fact that, with the exception of sulphanilamide (which was highly acetylated), they were hardly acetylated at all in this species (Marshall, 1945). Considering the large doses they used, Bieter *et al.* (1941) also obtained very low levels in the chick, and Litchfield (1939) found that a large proportion of sulphanilamide frequently passed through the gut of chicks without being absorbed. The foregoing facts make it obvious that the behaviour even of a group of drugs in any species is quite unpredictable. Unit dose per kgm. body weight has been used in all species and in going from the mouse to the large animals it clearly gives more comparable results than would be obtained by a dose based on metabolic rate. Specific metabolic differences appear to be more important than difference in metabolic rate, because, although the average B.L.C.A. produced in the cat is 1.6 times that in the mouse, the average B.L.C.A. in the fox is only 0.9 of that in the mouse; the average in the rabbit would clearly be much lower than in the mouse. The most interesting contrast is, however, provided by man, in whom the average B.L.C.A. to a dose of 4 gm. (approximately 0.06 gm./kgm. and therefore smaller than that given to the domestic animals) is nearly four times greater than that in the mouse and over twice as big as in the much heavier horse or cow.

*Behaviour of Individual Sulphonamides in Various Species.*-- The differing behaviour of each sulphonamide in the various species can best be seen by studying Fig. 12, in which the height of the columns corresponds to the B.L.C.A.s produced by the sulphonamides in different species (Table III).

When dealing with individual drugs the importance of specific metabolic differences between the species is even more evident than when dealing with the sulphonamides as a group. The results in the mouse resemble those in man as closely as do those in any other species. The main differences are that sulphadiazine produces relatively better and sulphathiazole and sulphanilamide relatively worse levels than in man. In contrast to the mouse sulphadiazine produces relatively poor levels in the domestic animals; in the calf the levels are actually worse than those produced by sulphathiazole. Despite the relatively small dose used in man it will be seen that higher levels were produced than in animals; indeed, those produced by sulphamerazine are outstanding. Four hours after administration the level is over twice as high as it would be if the drug had been absorbed and evenly distributed in the tissue without any being excreted, and it remains above this theoretical level for 24 hours. One assumes that this can only be due to failure of the drug to pass freely from the blood stream into the tissues. In confirmation

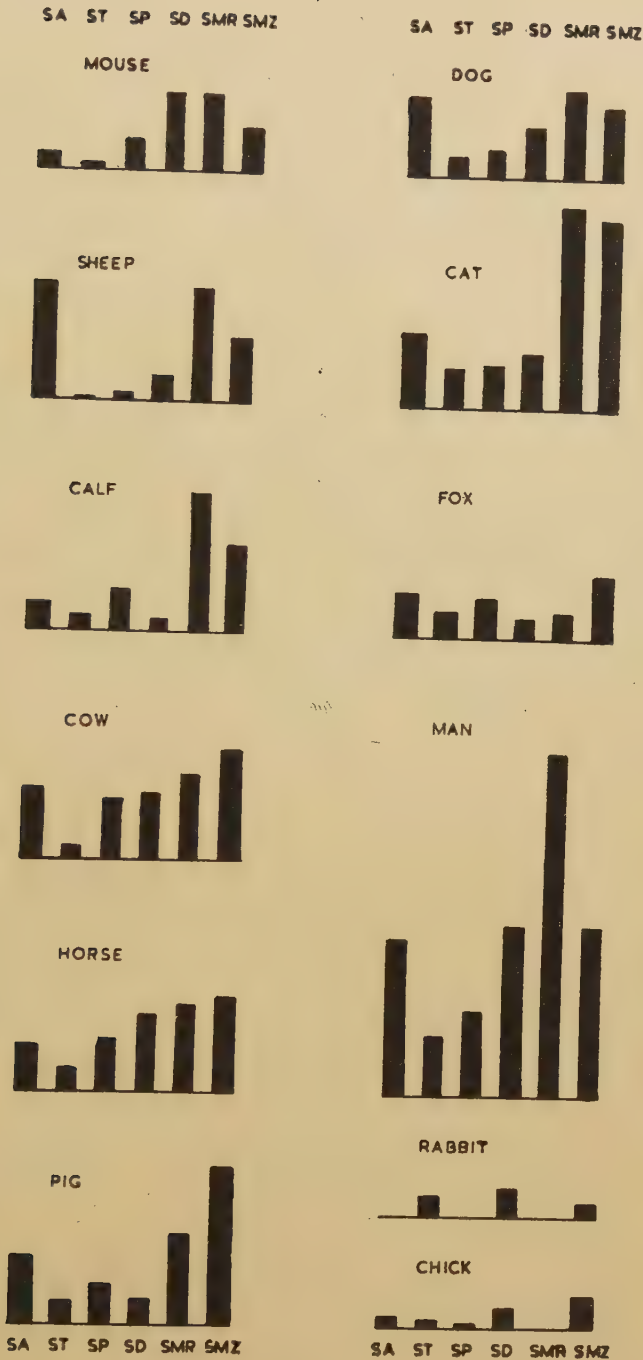


FIG. 12.—The average blood-level curve areas produced by the different sulphonamides in a variety of species.

*Footnote:* The data is comparative; in each instance the height of the column represents the blood-level curve area. For the method of adjusting the doses, see Table III and the text.

SA = Sulphanilamide; ST = Sulphathiazole; SP = Sulphapyridine; SD = Sulphadiazine; SMR = Sulphamerazine; SMZ = Sulphamezathine.



TABLE IV  
AVERAGE RELATIVE BLOOD-LEVEL CURVE AREAS PRODUCED BY THE INTRAVENOUS OR INTRAPERITONEAL INJECTION OF 0.05 GM./KGM. OF 4:4' DIAMINODIPHENYL SULPHONE AND VARIOUS SULPHONAMIDES IN THE PIG, CALF, AND SHEEP, TOGETHER WITH THE RATIO OF THE B.L.C.A.S PRODUCED BY THE ORAL DOSE\* TO THAT PRODUCED BY THE INTRAVENOUS OR INTRAPERITONEAL DOSE

| Animal | Dose<br>gm./kgm. | Sulphanilamide                           | Sulpha-<br>thiazole | Sulpha-<br>pyridine | Sulpha-<br>diazine | Sulpha-<br>merazine | Sulpha-<br>mezathine | 4:4'<br>diamino-<br>diphenyl<br>sulphone | Average |
|--------|------------------|------------------------------------------|---------------------|---------------------|--------------------|---------------------|----------------------|------------------------------------------|---------|
| Pig    | 0.05 i/p.        | Sulphanilamide in mouse<br>(oral) unity  | 1.2                 | 0.93                | 0.69               | 0.68                | 0.67                 | 3.4                                      | 1.3     |
|        |                  | Sulphanilamide in pig<br>(i/p) unity     | 1.0                 | 0.75                | 0.54               | 0.54                | 0.54                 | 2.7                                      | 1.1     |
|        |                  | Ratio oral*: i/p dose                    | 3.0                 | 1.1                 | 2.9                | 2.0                 | 6.1                  | 2.4                                      | 3.0     |
|        |                  |                                          |                     |                     |                    |                     |                      |                                          | 2.9     |
| Calf   | 0.05 i/v.        | Sulphanilamide in mouse<br>(oral) unity  | 0.53                | 0.7                 | 0.5                | 0.88                | 3.4                  | 2.3                                      | 3.9     |
|        |                  | Sulphanilamide in calf<br>(i/v) unity    | 1.0                 | 1.3                 | 0.94               | 1.7                 | 6.5                  | 4.3                                      | 7.3     |
|        |                  | Ratio oral*: i/v dose                    | 3.4                 | 1.0                 | 3.9                | 0.73                | 2.1                  | 2.2                                      | 3.0     |
|        |                  |                                          |                     |                     |                    |                     |                      |                                          | 2.3     |
| Sheep  | 0.05 i/v         | Sulphanilamide in mouse<br>(oral)* unity | 3.1                 | 0.35                | 0.55               | 1.0                 | 1.9                  | 1.75                                     | 2.6     |
|        |                  | Sulphanilamide in sheep<br>(i/v) unity   | 1.0                 | 0.11                | 0.17               | 0.33                | 0.6                  | 0.56                                     | 0.85    |
|        |                  | Ratio oral*: i/v dose                    | 2.0                 | 0.23                | 1.0                | 1.3                 | 3.2                  | 1.9                                      | 3.2     |
|        |                  |                                          |                     |                     |                    |                     |                      |                                          | 1.8     |

\* See Table III.

of this assumption it may be pointed out that Fisher *et al.* (1943) showed that the volume of distribution of sulphamerazine in the dog was 45·8 per cent. and it may be even less in man.

The dose given intravenously in the calf and sheep and intraperitoneally in the pig was half that given orally. The average ratio of the intravenous to oral B.L.C.A.s was 1:2·3 in both the calf and sheep. This is a smaller ratio than would have been obtained if 0·05 and 0·1 gm./kgm. doses had been given orally, but it has been found (Francis, 1947b) that higher blood-levels are obtained when a drug is injected intravenously than when it is given orally.

The rate of disappearance of drug from the blood was indicated by the size of the B.L.C.A. following intravenous injection. The shape of the curve and the size of the B.L.C.A. following oral dosing obviously depended on a combination of factors. The ratios between the B.L.C.A.s produced by oral and intravenous doses in the calf and sheep are shown in Table IV; if the ratio of intravenous to oral dosing with any drug was much less than the average ratio produced by the sulphonamide drugs as a group in any one species (*i.e.*, the intravenous level was relatively better than the oral) it seems reasonable to assume that the particular drug was poorly absorbed from the intestine. A converse result would indicate that the drug was relatively well absorbed.

Using these criteria, the behaviour of the various drugs can best be compared by examining Tables III and IV. With sulphathiazole the ratios of oral to intravenous or intraperitoneal dosing were low and strikingly so in the sheep, indicating that this drug was poorly absorbed. Jones (1947) showed that the kidney of the calf excreted sulphathiazole more rapidly than the other sulphonamides and the same is true in the dog (Beyer *et al.*, 1944). Sulphathiazole is also highly acetylated and this combination of factors accounts for the low levels produced in the mouse and domesticated animals. It produced relatively better levels in the chick, as it does in man. This may be due to the fact that, like the other heterocyclic sulphonamides, but not sulphanilamide, sulphathiazole is only very slightly acetylated in the chick, whereas it is highly acetylated in most animals.

In the horse sulphanilamide produced relatively good levels, but in general there was less difference between the individual drugs than in the other species studied. Niemand (1943) obtained results with sulphanilamide, sulphapyridine and sulphathiazole in the horse which were very similar to our own except that we did not obtain a high early peak of 8 mgm. per cent. with sulphanilamide. He found that sulphathiazole was minimally acetylated in the horse, which probably accounts for the relatively good levels it produced, sulphapyridine was slightly and sulphadiazine 8 to 20 per cent. acetylated.

The behaviour of sulphone calls for special comment. It has long been known that it produces methaemoglobinaemia in man,

guinea-pigs and mice and this is why it has not come into clinical use. It does not produce methaemoglobinaemia in sheep or cattle. In cattle it is less toxic than sulphanilamide, but in sheep it is very liable to produce toxic nervous effects rather similar to those described in man following an overdose of sulphanilamide (Reed, 1944). It has already been mentioned that at one stage after dosing nearly 70 per cent. methaemoglobin is produced in the horse, and we have also observed that it produces quite severe methaemoglobinaemia in the pig and dog. The mucous membranes of the horses were of a chocolate colour, but there was no evidence of cyanosis or respiratory distress, whereas Bodansky and Gutmann (1947) state that 60 per cent. methaemoglobinaemia may cause ataxia and salivation in the dog. They showed, however, that although the administration of methylene blue caused a rapid abatement of severe and fatal symptoms, it only produced a very slight effect on the methaemoglobin level.

*Effect of Age on the Behaviour of the Sulphonamides in the Bovine.*—Jones (1947) found that sulphathiazole produced relatively better levels in young than old calves. He also reported that sulphapyridine was more slowly absorbed and disappeared much more slowly from the blood in young than in old calves. The relatively good levels obtained with sulphathiazole in the present study were probably due to the use of very young calves. The results obtained in cows and calves (Figs. 6, 9 and 13 and Table III) show that the same age phenomenon occurred with sulphone. It will be seen from Fig. 13 that a single dose of 0.1 gm./kgm. produced a maximum level of 3.5 mgm. per cent. in the calf, which remained at above 1 mgm. per cent. for four days. The same dose in the cow produced a maximum level of 6.5 mgm. per cent., but all the drug

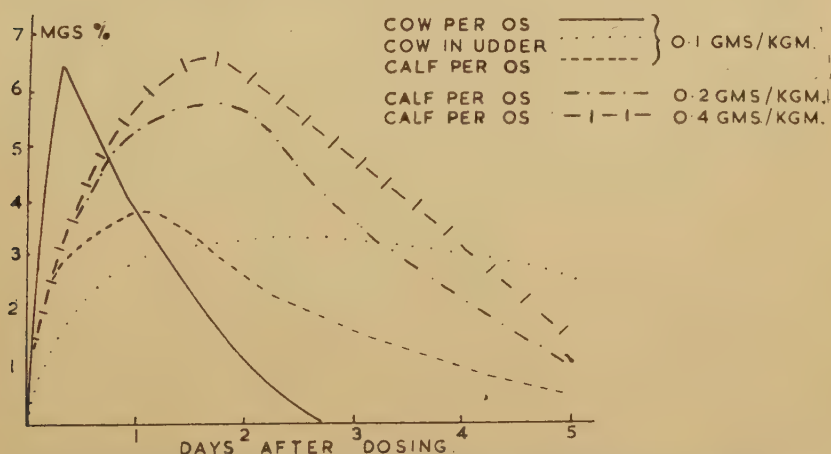


FIG. 13.—Average blood-levels produced in the cow and the calf by administration of various doses of sulphone and sulphanilamide orally or into the udder.

had disappeared within two and a half days. When a dose of 0.1 gm./kgm. suspended in oil was injected into the non-lactating udder a maximum blood-level of about 3 mgm. per cent. was produced in two to three days and the drug could be detected in the blood for 12 to 16 days (Francis, 1947b). These results indicate that the difference in behaviour between the cow and the calf was due to differences in the rate of absorption, because the rate of disappearance from the blood, at any given level, is presumably the same whether a drug is given orally or injected into the udder. In general there was much less difference in the behaviour of the various sulphonamides in the cow than in the calf; sulphanilamide produced relatively and absolutely much worse, and sulphone much better, levels in the calf than in the cow.

The different behaviour of the sulphonamides in the calf and cow is of considerable interest. Peterson *et al.* (1942) showed that some of the sulphonamides were absorbed less well following duodenal than oral administration, whilst others were absorbed equally well following either method of administration. Greater changes occur in the intestinal tract of the ruminant than in other animals. In the adult the rumen is well developed, ingested fluids pass into it and there can be little doubt that the sulphonamides are absorbed from it. In the calf fluids usually pass to the abomasum *via* the oesophageal groove and it is probably these changes which account for the differences in absorption in the cow and calf.

The present study was undertaken largely to obtain data which would be of practical value in veterinary medicine. It has, however, brought out the danger of transferring data uncritically from one species to another. For instance, in discussing their results in the dog, Beyer *et al.* (1944) point out that if protein binding and tubular resorption were the limiting factors in determining elimination of a drug, the level of "Sulphamezathine" should be better maintained than that of the other compounds. They then say, on the basis of results in man "it is well known that the blood-level of sulfamethazine is not sustained as well by single doses of the drug as is the case for sulfamerazine and sulfadiazine." Their own evidence suggests that this statement would be untrue if applied to the dog and the results in Fig. 12 show that it is erroneous.

#### SUMMARY

Blood-levels following the oral administration of 0.1 gm./kgm. of sulphanilamide, sulphathiazole, sulphapyridine, sulphadiazine, sulphamerazine, "Sulphamezathine," and 4:4'diaminodiphenyl sulphone to pigs, horses, sheep and calves were determined. Blood-levels following the intravenous injection of 0.05 gm./kgm. of the same drugs in calves and sheep and the intraperitoneal injection in pigs were determined.

The same drugs were also administered orally to sheep at a dose of 0.2 gm./kgm. From these results and other evidence in the litera-

ture it is concluded that doubling the dose of the sulphonamide drugs (over this range) trebled the blood-level curve area (B.L.C.A.). Using this conversion factor our own results have been compared with those of other workers who used slightly different doses and it has thus been possible to include data on a variety of species.

Blood- and milk-levels following the oral administration of 0.1 gm./kgm. of sulphanilamide and 4:4'diaminodiphenyl sulphone to cows were determined. Some observations were also made on the behaviour of 4-nitro:4'-aminodiphenyl sulphone and on acetylation of the sulphonamides.

The average B.L.C.A.s produced by the seven drugs in the pig, horse, calf and sheep, when 0.1 gm./kgm. was administered orally, were very similar, but the pig absorbed the drug considerably more rapidly than the calf and sheep, in which animals the average curves were very similar. The same dose given to mice and chicks produced smaller B.L.C.A.s.

There were striking differences in the behaviour of the individual drugs in the different species and by comparing the levels obtained following intravenous or intraperitoneal injection and oral dosing, an indication was obtained as to whether this was due to poor absorption or rapid disappearance from the blood. 4:4'diaminodiphenyl sulphone produced the best levels in all animals, except the pig, in which its place was taken by "Sulphamezathine" which was well absorbed and slowly excreted. 4:4'diaminodiphenyl sulphone produced methaemoglobinaemia in the mouse, pig and horse, but not in the sheep or calf; it was less toxic than sulphanilamide in the bovine, but fairly readily produced toxic nervous effects in the sheep.

Judging from data in the literature the sulphonamides produce considerably higher levels in man than in any other animal studied.

Sulphanilamide produced good blood-levels in the cow and very poor levels in the calf. On the other hand, 4:4'diaminodiphenyl sulphone produced better levels in calves than cows. This interesting effect of age was probably chiefly due to development of the rumen and consequent effects on absorption.

In general the sulphonamides producing good levels of the free drug in the calf were only moderately acetylated and those producing poor levels much more acetylated.

This study has demonstrated the danger of transferring data uncritically from one species to another.

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METHODS FOR THE RECOVERY OF *STR. AGALACTIAE*  
FROM OUTSIDE THE BOVINE UDDER

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## INTRODUCTION

MANY workers have shown that *Str. agalactiae* can be isolated from the outside of the teats, from milkers' hands and from various sites which are exposed to contamination with infected milk. The demonstration of the organism is, however, often difficult and various selective media have been described. Those most commonly used have contained crystal violet alone (Bull *et al.*, 1940; Harrison, 1941) or in combination with other substances (Spencer *et al.*, 1946; and Report, 1944). McKenzie (1941) reported the value of thallium acetate in liquid media for the recovery of *Str. agalactiae* from milk samples, whilst Fleming (1932) and Fleming and Young (1940) used potassium tellurite for the isolation of streptococci from heavily contaminated material.

We became interested in this question during the course of some experiments on the transmission of *Str. agalactiae* from cow to cow by milking with hands which had been dipped in *Str. agalactiae* infected milk (Lancaster and Stuart 1949), when we wished to find out how long *Str. agalactiae* remained viable on the surface of the teats. Although it was known that the teats had been recently heavily infected with *Str. agalactiae*, the recovery of the organisms from these sites was at times impossible or very difficult, especially if the teats had become splashed with faeces. Attempts were, therefore, made to find a satisfactory method of recovery of *Str. agalactiae* from highly contaminated places of this kind, and also from other sites on the cow and in the cowshed.

## METHODS

Swabs, prepared by tightly wrapping a piece of absorbent cotton wool around the end of a piece of stout glass rod, about 5 to 6 in. long, were placed in 7 in.  $\times$  1 in. glass tubes, which were then plugged and sterilised by autoclaving. The swab was moistened in sterile distilled water, saline or broth, rubbed over the area to be tested and then agitated in a 4½ in.  $\times$  5/8 in. tube containing 5 ml. of either glucose broth, serum broth, sterile milk alone, or sterile milk with the addition of dye. After agitation, the excess liquid was pressed out of the swab and the latter discarded. The tube of medium was incubated for 18 hours at 37° C. and a loopful of broth or, in the case of milk, of cream was then streaked on one-third of the surface of a sheep blood nutrient agar plate containing final concentrations of aesculin 1/1,000 and crystal violet 1/500,000

(Edwards, 1933) and thallium acetate 1/2,000.\* Using the above method, swabs were taken from the skin of the udder and teats, the cowshed fittings, the floor and the cleansing and dairy utensils. These preliminary trials indicated that milk was the most suitable basic medium. Tests were, therefore, made with sterile milk, with and without various dye additions.

## RESULTS

*Effect of adding dyes to medium*

A large number of experiments have been done, those now being reported being representative of the larger series.

*Experiment 1.*—A quantity of whole fresh milk was sterilised by autoclaving, and the required volumes of milk or milk and dyes placed in sterile tubes as shown in Table I.

TABLE I

| Tube No. | Volume of sterile milk | Volume of stock dye media added to each tube |                      |                        |                           | Final concentration of dyes (approx.) |                 |                   |                                |
|----------|------------------------|----------------------------------------------|----------------------|------------------------|---------------------------|---------------------------------------|-----------------|-------------------|--------------------------------|
|          |                        | Potas-<br>sium<br>tellurite                  | Bor-<br>acic<br>acid | Cry-<br>stal<br>violet | Thal-<br>lium<br>sulphate | Potassium<br>tellurite                | Boracic<br>acid | Crystal<br>violet | Thal-<br>lium<br>sul-<br>phate |
|          |                        | 0.1<br>%                                     | 4<br>%               | 1 : 25,000             | 1 : 100                   |                                       |                 |                   |                                |
|          | ml.                    | ml.                                          | ml.                  | ml.                    | ml.                       |                                       |                 |                   |                                |
| 1        | 5                      | —                                            | —                    | —                      | —                         | —                                     | —               | —                 | —                              |
| 2        | 4.5                    | 0.25                                         | 0.25                 | —                      | —                         | 1 : 20,000                            | 1 : 500         | —                 | —                              |
| 3        | 3.75                   | 0.5                                          | 0.25                 | 0.5                    | —                         | 1 : 10,000                            | 1 : 500         | 1 : 250,000       | —                              |
| 4        | 4.0                    | 0.25                                         | 0.25                 | —                      | 0.5                       | 1 : 20,000                            | 1 : 500         | —                 | 1 : 1,000                      |
| 5        | 4.0                    | —                                            | 0.25                 | 0.5                    | 0.25                      | —                                     | 1 : 500         | 1 : 250,000       | 1 : 2,000                      |
| 6        | 4.0                    | —                                            | 0.5                  | —                      | 0.5                       | —                                     | 1 : 250         | —                 | 1 : 1,000                      |

A mixed culture of various organisms (for ease of reference these will be termed contaminants) found in the cow byre and on the skin of cows was prepared in sterile whole milk. No attempt was made to identify the various organisms present. A pure culture of *Str. agalactiae* was also prepared in milk. Tenfold dilutions of the contaminants and *Str. agalactiae* were made separately in sterile milk and viable plate counts made. Sets of tubes containing a tube of sterilised milk and one of each of the dye media as listed in Table I were inoculated with different proportions of *Str. agalactiae* and contaminants. *e.g.*, the first series contained four million *Str. agalactiae* and 300,000 to < 3 contaminants. In each of the subsequent series the numbers of streptococci were reduced tenfold until series six contained four *Str. agalactiae* and 300,000 to < 3 contaminants (Fig. 1). The tubes were then incubated at 37° C. After 18 hours, 48 hours and 72 hours, a loopful of the cream was taken and spread over the surface of one-third of a dye plate, which was incubated at 37° C. for 48 hours.

\* Referred to subsequently as the dye plate. Samples of each batch of inhibitory chemicals and prepared medium should be tested before use with known cultures, in order to check the proper concentration, efficiency and sterility.

The results which are shown in Fig. 1 indicate that, when the initial ratio of contaminants to *Str. agalactiae* is low, then sterile milk alone forms the best medium for the subsequent recovery of *Str. agalactiae*. This is because all the dyes used have an inhibitory effect on the growth of *Str. agalactiae*, which is specially noticeable

FIG. 1  
RESULTS AFTER 18 HOURS' INCUBATION OF THE TUBES AND PLATING  
(Experiment 1)

| Number of viable<br>contaminants im-<br>planted in tube | Dye<br>tube<br>No. | Number of viable <i>Str. agalactiae</i> implanted in tube |         |        |       |     |    |   |
|---------------------------------------------------------|--------------------|-----------------------------------------------------------|---------|--------|-------|-----|----|---|
|                                                         |                    | 4,000,000                                                 | 400,000 | 40,000 | 4,000 | 400 | 40 | 4 |
| 300,000                                                 | 1                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 2                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 3                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 4                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 5                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 6                  | _____                                                     |         |        |       |     |    |   |
| 30,000                                                  | 1                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 2                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 3                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 4                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 5                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 6                  | _____                                                     |         |        |       |     |    |   |
| 3,000                                                   | 1                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 2                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 3                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 4                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 5                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 6                  | _____                                                     |         |        |       |     |    |   |
| 300                                                     | 1                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 2                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 3                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 4                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 5                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 6                  | _____                                                     |         |        |       |     |    |   |
| 3                                                       | 1                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 2                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 3                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 4                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 5                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 6                  | _____                                                     |         |        |       |     |    |   |
| <3                                                      | 1                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 2                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 3                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 4                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 5                  | _____                                                     |         |        |       |     |    |   |
|                                                         | 6                  | _____                                                     |         |        |       |     |    |   |

The horizontal lines "\_\_\_\_\_" indicate those inocula of *Str. agalactiae* which, in the presence of the numbers of contaminants shown, were recovered by means of the various dye media.

when the latter is present in small numbers. When, however, the initial number of contaminants exceeds that of *Str. agalactiae*, dye media are necessary and with moderate or high ratios of contaminants, medium No. 2 appeared to be one of the most suitable of the series.

The value of the potassium tellurite and boracic acid mixture in the recovery of *Str. agalactiae* from contaminated material was further emphasised in subsequent experiments.

*Experiment II.*—Tubes containing 5 ml. of sterile whole milk were prepared and to these were added various dyes. One half ml. of a mixture consisting of *Str. agalactiae* infected milk, contaminants from cows' skin and dung was then added to each tube of dye medium. After incubation for 15 hours at 37° C, a loopful of the cream was plated on the surface of one half of a dye plate and the results, recorded as in Experiment I, are shown in Table II.

TABLE II  
FINAL CONCENTRATION OF DYES CONTAINED IN 5 ML. MILK AND RESULTS

| Tube No. | Potassium tellurite | Boracic acid | Crystal violet | Thallium sulphate | Sodium azide | Acridine flavine | Number of visible <i>Str. agalactiae</i> colonies | Number of contaminants |
|----------|---------------------|--------------|----------------|-------------------|--------------|------------------|---------------------------------------------------|------------------------|
| 1        | —                   | —            | —              | —                 | —            | —                | 0*                                                | ++++*                  |
| 2        | 1:20,000            | —            | —              | —                 | —            | —                | +++                                               | ++                     |
| 3        | 1:10,000            | —            | —              | —                 | —            | —                | +++                                               | ++++                   |
| 4        | —                   | 1:500        | —              | —                 | —            | —                | +++++                                             | ++++                   |
| 5        | —                   | 1:1,250      | —              | —                 | —            | —                | +                                                 | +++++                  |
| 6        | —                   | —            | 1:500,000      | —                 | —            | —                | +                                                 | ++                     |
| 7        | —                   | —            | 1:1,250,000    | —                 | —            | —                | +                                                 | ++                     |
| 8        | —                   | —            | —              | 1:1,000           | —            | —                | +                                                 | ++++                   |
| 9        | 1:20,000            | 1:1,250      | —              | —                 | —            | —                | ++++                                              | +                      |
| 10       | 1:20,000            | 1:500        | —              | —                 | —            | —                | ++++                                              | 0                      |
| 11       | 1:20,000            | 1:312        | —              | —                 | —            | —                | +                                                 | ++                     |
| 12       | 1:20,000            | 1:500        | 1:500,000      | —                 | —            | —                | ++++                                              | +                      |
| 13       | —                   | —            | 1:1,250,000    | 1:2,000           | —            | —                | ++                                                | ++                     |
| 14       | —                   | —            | —              | —                 | 1:10,000     | —                | 0                                                 | +++++                  |
| 15       | —                   | —            | —              | —                 | 1:20,000     | —                | +                                                 | ++++                   |
| 16       | —                   | —            | 1:500,000      | —                 | 1:20,000     | —                | +                                                 | ++++                   |
| 17       | —                   | —            | —              | —                 | —            | 1:200,000        | +++++                                             | ++++                   |
| 18       | 1:20,000            | —            | —              | —                 | —            | 1:200,000        | +++                                               | +                      |

\*0 Means no visible growth  
 + " 1-50 colonies  
 ++ " 50-100 colonies  
 +++ " 100-500 colonies  
 ++++ " > 500 colonies

*Experiment III.*—Swabs moistened in sterile milk were rubbed on the teats of four cows, then agitated and squeezed into four McCartney bottles containing 20 ml. of sterile milk (Tube No. 1). A separate swab and McCartney bottle was used for each cow.

After mixing, 0.5 ml. of milk from each bottle was transferred to each of five tubes of dye media (Tube Nos. 2 to 6). These were incubated, plated and examined as in Experiment II.

From the above experiments (Table III) it was concluded that potassium tellurite and boracic acid appeared the most suitable substances for the preparation of the liquid selective medium required.

TABLE III

| COW | Final concentration of dyes contained in 5 ml. milk |          |                | Cow 756 |      | Cow 767 |      | Cow 928 |      | Cow 930 |     |
|-----|-----------------------------------------------------|----------|----------------|---------|------|---------|------|---------|------|---------|-----|
|     | Potassium tellurite                                 | Bor-acid | Crystal violet |         |      |         |      |         |      |         |     |
|     | (i.e., 5 ml. sterile milk)                          |          |                | *       | *    |         |      |         |      |         |     |
| 1   | —                                                   | —        | —              | 0       | ++++ | +       | ++++ | ++      | +++  | ++++    | +++ |
| 2   | 1: 20,000                                           | —        | —              | 0       | ++++ | +       | ++++ | 0       | ++++ | ++++    | ++  |
| 3   | —                                                   | 1: 500   | —              | 0       | ++++ | ++++    | +    | ++++    | ++   | ++++    | +   |
| 4   | —                                                   | —        | 1: 500,000     | 0       | ++++ | 0       | ++++ | 0       | ++++ | ++++    | ++  |
| 5   | 1: 20,000                                           | 1: 500   | —              | ++      | ++++ | ++++    | ++   | ++++    | +    | ++++    | 0   |
| 6   | 1: 20,000                                           | 1: 500   | 1: 500,000     | +       | ++++ | ++++    | ++++ | ++++    | ++   | ++++    | +   |

\* The first column under each cow represents the number of visible *Str. agalactiae* colonies; the second column represents the number of contaminants. See Key, Table II.

*Experiment IV.*—A comparison was then made of the present method and that described by another group of workers in England (Report, 1944). In this method sterile swabs moistened in sterile tap water and rubbed on the teats of the cow were agitated and squeezed into 10 ml. of sterile milk in a McCartney bottle. The milk was incubated overnight at 37° C., and, after shaking, a loopful of the mixed milk was streaked on a dye plate consisting of sheep blood agar containing final concentrations of aesculin 1/1,000, crystal violet 1/500,000 and thallium acetate 1/2,000. These plates were incubated 48 hours at 37° C. and the number of visible *Str. agalactiae* colonies and the number of contaminants were recorded. This will be referred to as the "sterile milk method."

For the sake of clarity our own method at this date will be briefly recapitulated. The swab, after moistening in sterile milk and rubbing on the teat of the cow, was agitated and squeezed into 5 ml. of sterile milk, containing potassium tellurite 1/20,000 and boracic acid 1/500, final concentrations. The inoculated dye media were then incubated for 18 hours at 37° C. and examined by streaking a loopful of the cream on the surface of a dye plate. The plates were incubated 48 hours at 37° C. and the results recorded as in Experiment I.

Duplicate swabs were taken from the teats of all 16 cows in the laboratory experimental herd. For cow No. 1 the sterile milk

method was used first, then the present method. For cow No. 2 the present method was used first, followed by the sterile milk method, and so on.

TABLE IV

| Number<br>of<br>cow | Lab.<br>number | Present method              |              | Sterile milk method |              |
|---------------------|----------------|-----------------------------|--------------|---------------------|--------------|
|                     |                | Pot. tell. and boracic acid |              |                     |              |
|                     |                | Str. agalactiae             | Contaminants | Str. agalactiae     | Contaminants |
| 1                   | 930            | ++                          | 0            | 0                   | +++++        |
| 2                   | 929            | +++++                       | 0            | 0                   | +++++        |
| 3                   | 928            | +++++                       | 0            | ++                  | +++++        |
| 4                   | 759            | ++                          | +            | 0                   | +++++        |
| 5                   | 758            | +++++                       | +            | 0                   | +++++        |
| 6                   | 756            | +                           | +++          | 0                   | +++++        |
| 7                   | 766            | +++                         | +            | 0                   | +++++        |
| 8                   | 767            | +                           | +++          | 0                   | +++++        |
| 9                   | 968            | +++                         | 0            | 0                   | +++++        |
| 10                  | 967            | +++                         | +            | 0                   | +++++        |
| 11                  | 966            | 0                           | ++           | 0                   | +++++        |
| 12                  | 965            | +                           | ++           | +++++               | +++          |
| 13                  | 964            | +++                         | +            | 0                   | +++++        |
| 14                  | 963            | 0                           | +++          | 0                   | +++++        |
| 15                  | 962            | ++                          | 0            | 0                   | +++++        |
| 16                  | 970            | ++                          | ++           | 0                   | +++++        |

For interpretation, see key attached to Table II

Table IV shows that, with the present method, it was not only possible to recover *Str. agalactiae* from 14 samples out of 16 (88 per cent.), but that from five samples the streptococci were obtained in pure culture, the contaminating organisms being completely inhibited; while in the remainder of the plates, the number of contaminants was generally low. The sterile milk method gave only two positive cows (15 per cent.), while on almost all plates the number of contaminants exceeded 500 colonies.

#### *Recovery of the Str. agalactiae from the air of the cowshed*

A sterile Petri dish lid (4 in. diameter) was inverted and filled with 30 ml. sterile whole milk and protected from cats, vermin, etc., by an inverted, previously sterilised wire crate ( $\frac{1}{4}$  in. square mesh). These Petri dishes were exposed for 6 to 20 hours in various parts of the cowshed. After exposure, 5 ml. of the milk was transferred from the Petri dish to each of five tubes containing dyes to provide the concentration shown in Table V. The inoculated tubes were then incubated overnight and a loopful of cream streaked on to one-third of a dye plate. The results were recorded after 48 hours' incubation at 37°C. This experiment suggests that tubes 1, 3 and 5 contained the most suitable media. Subsequent tests indicated that of these three media, that containing boracic acid 1/500, crystal violet 1/500,000 and thallium sulphate 1/2,000 was the best.

TABLE V

| Tube No. | Final concentration of dyes contained in 5 ml. milk |              |                |                   | Four Petri dishes exposed simultaneously for six hours during daytime in various sites in cow byre |      |      |   | Four Petri dishes exposed simultaneously for 18 hours during night-time in various sites in cow byre |   |    |   |
|----------|-----------------------------------------------------|--------------|----------------|-------------------|----------------------------------------------------------------------------------------------------|------|------|---|------------------------------------------------------------------------------------------------------|---|----|---|
|          |                                                     |              |                |                   |                                                                                                    |      |      |   |                                                                                                      |   |    |   |
|          | Potassium tellurite                                 | Boracic acid | Crystal violet | Thallium sulphate | Petri dish No.                                                                                     |      |      |   | Petri dish No.                                                                                       |   |    |   |
|          |                                                     |              |                |                   | 1                                                                                                  | 2    | 3    | 4 | 1                                                                                                    | 2 | 3  | 4 |
| 1        | { (i.e., 5 ml. sterile milk)                        |              |                |                   | —                                                                                                  | ++++ | +    | + | +                                                                                                    | + | 0  | 0 |
| 2        | 1: 20,000                                           | 1: 500       | —              | —                 | —                                                                                                  | ++   | 0    | 0 | 0                                                                                                    | 0 | ++ | 0 |
| 3        | 1: 20,000                                           | 1: 500       | 1: 500,000     | —                 | —                                                                                                  | ++   | 0    | + | 0                                                                                                    | + | +  | 0 |
| 4        | 1: 20,000                                           | 1: 500       | —              | 1: 2,000          | +++                                                                                                | 0    | ++++ | 0 | 0                                                                                                    | 0 | 0  | 0 |
| 5        | —                                                   | 1: 500       | 1: 500,000     | 1: 2,000          | ++                                                                                                 | +    | +    | + | ++                                                                                                   | 0 | +  | 0 |

In the above table + indicates 1-50 colonies of *Str. agalactiae*  
 ++ " 50-100 " " "  
 +++ " 100-500 " " "  
 ++++ " above 500 " " "

For these observations, the Petri dishes exposed during the night were located in the same position in the cow byre as those exposed during daytime.

#### *Recovery of Str. agalactiae from the faeces on the floor of the cow byre*

Using a dry sterile swab, a small quantity of faeces was taken separately from each cow and mixed in 8 ml. sterile milk. This mixture was distributed in 0.5 ml. amounts to 12 tubes of 5 ml. sterile milk containing various dye media. These tubes were then incubated and examined as for air sampling or teat swabbing. The best of this series of dye media appeared to be potassium tellurite 1/20,000, boracic acid 1/500 and crystal violet 1/500,000 final concentrations. Using this mixture, *Str. agalactiae* was recovered from faeces of three cows out of 20.

It is not suggested that these results indicate the presence of *Str. agalactiae* in the intestinal tract. In view of the finding of *Str. agalactiae* in the air, the presence of this organism in the faeces is more likely to be the result of aerial contamination.

#### *Recovery of Str. agalactiae from milker's hands*

The hands were first rinsed in tap water, then washed and brushed in sterile milk according to the method of Harrison (1941). Milk was drawn from the basin into a sterile flask, brought to the laboratory and distributed in 5 ml. amounts to three tubes, two of which contained dyes to give the final concentrations as indicated in Table VI. The tubes were then incubated and examined as previously described.

The information in Table VI indicates that, when the milker's hands are heavily contaminated, the use of dye media is necessary for the recovery of *Str. agalactiae*.

TABLE VI

| Milker 5 ml. milk hand washings |                    | 5 ml. milk hand washings containing final concentrations of the following dyes |                    |                                                                                      |                    |                   |
|---------------------------------|--------------------|--------------------------------------------------------------------------------|--------------------|--------------------------------------------------------------------------------------|--------------------|-------------------|
|                                 |                    | Potassium tellurite,<br>1 : 20,000; and boracic<br>acid, 1 : 500               |                    | Boracic acid, 1 : 500;<br>crystal violet 1 : 500,000;<br>thallium sulphate 1 : 2,000 |                    |                   |
|                                 | Str.<br>agalactiae | Con-<br>taminants                                                              | Str.<br>agalactiae | Con-<br>taminants                                                                    | Str.<br>agalactiae | Con-<br>taminants |
| 1                               | 0                  | ++++                                                                           | ++                 | +++                                                                                  | ++                 | ++                |
| 2                               | 0                  | ++++                                                                           | ++                 | +                                                                                    | ++                 | ++                |
| 3                               | 0                  | ++++                                                                           | +                  | +                                                                                    | +                  | ++                |
| 4                               | 0                  | ++++                                                                           | 0                  | +                                                                                    | 0                  | +                 |
| 5                               | 0                  | ++++                                                                           | ++                 | ++                                                                                   | +                  | +++               |

For interpretation, see key attached to Table II.

### Modified Method

In the work so far described milk, or milk and dye media, was incubated overnight and a loopful of cream plated on the solid selective medium. In the next series of experiments, swabs were taken in the usual way and agitated in the best selective medium so far found (potassium tellurite 1/20,000 and boracic acid 1/500) and explants made on to dye plates comprising sheep blood nutrient agar containing crystal violet 1/750,000, thallium sulphate 1/3,000 and aesculin 1/1,000 after incubation for 5, 15 and 30 minutes and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 24 hours. It was found that much better results were obtained at or around 2 or 3 hours than at any other period. The reason may be that *Str. agalactiae* starts to multiply earlier than most other organisms (Steck, 1934), that it is inhibited to some extent by long incubation in the selective medium concerned and that many contaminants overgrow it if there is a long period of incubation before plating. In the course of the above experiments a further slight modification of the solid medium used for plating was also tested. It was found that somewhat better results were obtained by reducing the crystal violet to 1/750,000 and replacing thallium acetate at 1/2,000 with thallium sulphate at 1/3,000.

### FINAL COMPARISON OF METHODS

Tests were made on five farms to compare the Modified Method just described with that adopted previously. In these tests one swab was taken from each site and agitated in a tube containing the above dye milk medium. This tube was brought to the laboratory and after incubation for 2 to 3 hours at 37° C., 0.1 ml. of whole milk from the more contaminated sources, *e.g.*, cow's skin, cowshed floor, cowshed cleaning utensils, etc., was spread on the surface of the modified dye plate. In the case of the less contaminated sources, *e.g.*, clean dairy utensils, hands, etc., the

amount of milk spread was 0.3 ml. The remaining milk in the tube was then incubated for a further 18 hours and one loopful of cream streaked on to part of the surface of a dye plate (original formula). The plates were incubated 48 hours at 37° C. and read (Table VII). In all these tests colonies regarded as *Str. agalactiae* were confirmed serologically. It will be seen that the modified method gave a much larger number of positive results than the original method.

TABLE VII

| Source of material | Teats           |                 | Skin of cow (abdomen, thighs, etc.) |                 | Cowshed and utensils |                 | Dairy and utensils and milkers' clothes |                 | Hand washings   |                 |
|--------------------|-----------------|-----------------|-------------------------------------|-----------------|----------------------|-----------------|-----------------------------------------|-----------------|-----------------|-----------------|
|                    | Original method | Modified method | Original method                     | Modified method | Original method      | Modified method | Original method                         | Modified method | Original method | Modified method |
|                    | 22/151          | 77/151          | 2/151                               | 6/151           | 0/130                | 11/130          | 1/81                                    | 6/81            | 1/12            | 4/12            |

Key: The numerator indicates the number of *Str. agalactiae* positive samples.  
The denominator indicates the number of samples taken.

#### *Use of the Modified Selective Solid Medium for Milk Samples*

The modified selective solid medium consisting of sheep's blood nutrient agar plate containing crystal violet 1/750,000, aesculin 1/1,000 and thallium sulphate 1/3,000 has also been found to be a useful medium for the examination of milk samples. The plates keep well in the cold. The presence of the dyes does not appear to inhibit greatly the number and size of the streptococcal colonies when compared with plain sheep blood nutrient agar plates. The appearance of the colonies of *Str. agalactiae* and *Str. dysgalactiae* on the medium is characteristic, *Str. agalactiae* giving haemolytic or non-haemolytic bluish colonies with no discoloration of the medium, *Str. dysgalactiae* appearing as greyish colonies producing a yellowish green discoloration. Coliform organisms, moulds, staphylococci and diphtheroids are inhibited. The medium might prove valuable in routine work.

#### SUMMARY

Experiments have been made in order to find suitable methods for the recovery of *Str. agalactiae* from sources outside the bovine udder.

The method finally adopted for sites other than faeces, air and hands is as follows. The area is swabbed and the swab is agitated in a tube of sterilised milk containing potassium tellurite 1/20,000 and boracic acid 1/500 as final concentrations. After 2 to 3 hours' incubation at 37° C., 0.1 to 0.3 ml. (according to site) is spread on a sheep blood agar plate containing crystal violet 1/750,000, aesculin 1/1,000 and thallium sulphate 1/3,000; the results are read after 48 hours at 37° C.

Incubation for shorter periods or for periods up to 12 hours or the use of a loopful of gravity cream after overnight incubation were found inferior.

Methods are also described for hands, faeces and air.

The solid medium described is useful for the primary isolation and identification of *Str. agalactiae* or *Str. dysgalactiae* from fresh or contaminated milk samples.

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THE DISTRIBUTION OF *STR. AGALACTIAE* OUTSIDE THE BOVINE UDDER AND ITS SURVIVAL

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## INTRODUCTION

It has long been known that the main reservoir of *Str. agalactiae* is the bovine udder and its secretion. Recent work has indicated, however, that this organism can commonly be isolated from the outside of the teats, udder or other parts of the skin and from utensils and various parts of the cowshed, and these sites may provide a source of infection. This aspect of the disease becomes of special importance when the eradication of the disease from whole herds is the goal.

The presence and survival of *Str. agalactiae* outside the bovine udder has been dealt with previously by several workers. Klingmuller (1930) found that in 40 per cent. of tubes containing straw and bedding which had been inoculated with *Str. agalactiae* viable organisms were recovered after three and a half months. Bryan (1934) recovered *Str. agalactiae* in the litter and in the dust of dairy barns 20 to 30 days after removing the infected animals, in sterile sand after 66 days, in sterile soil after nine days and in tap water after 65 days. Bull *et al.* (1940) isolated *Str. agalactiae* from the lesions (sores and cracks) of the teats and udder and from the faeces of seven cows. According to Harrison (1941), *Str. agalactiae* may be present for many days on the hands of persons who have been in contact with infected milk. Hay (1941) recovered *Str. agalactiae* from milking machine cups. Watts (1941) reported that *Str. agalactiae* can survive outside the animal body for several months, but gives no details. A group of British workers (Report, 1944), reported the recovery of *Str. agalactiae* from normal and abraded teats, from the air of the cowshed, from the churn handles, broom handles, door knob, etc. Spencer *et al.* (1946) demonstrated *Str. agalactiae* as resident flora on milker's hands. They also found that these organisms were usually dead in bedding after 24 hours, but a few survived up to nine days.

In the present paper further work on these problems is reported using the Modified Method and selective liquid and solid media which have been described in a previous paper (Chodkowski and Lancaster, 1949). Colonies suspected as *Str. agalactiae* were identified by precipitin test in all doubtful cases and in a large proportion of other cases.

THE RECOVERY OF *Str. agalactiae* FROM VARIOUS SITES ON THE  
OUTSIDE OF THE COW'S SKIN, FROM VARIOUS PARTS OF THE COWSHED  
AND UTENSILS IN INFECTED HERDS

The first observations (Table I) were made in the laboratory herd during the course of a transmission experiment in which the animals were being milked with infected hands, swabs being taken six to 16 hours after milking. This circumstance accounts for the high percentage of positive results from the teats and skin of the udder. It will be seen that *Str. agalactiae* was recovered also from a high percentage of various other sites on the cows, as well as from utensils, the floor, air and workers' boots.

TABLE I

| Site                      | Number of<br>swabs taken | Number of<br>swabs positive | Percentage<br>swabs positive |
|---------------------------|--------------------------|-----------------------------|------------------------------|
| <i>A. Cow</i>             |                          |                             |                              |
| Teats ... ..              | 96                       | 90                          | 94                           |
| Udder ... ..              | 4                        | 4                           | (100)                        |
| Nostrils ... ..           | 19                       | 1                           |                              |
| Mouth ... ..              | 19                       | 1                           |                              |
| Massetter muscle skin ... | 10                       | 2                           | 20                           |
| Neck ... ..               | 10                       | 0                           |                              |
| Chest ... ..              | 84                       | 21                          | 25                           |
| Shoulders ... ..          | 84                       | 28                          | 33                           |
| Flank ... ..              | 89                       | 34                          | 38                           |
| Abdomen ... ..            | 12                       | 4                           | 33                           |
| Thighs ... ..             | 91                       | 38                          | 42                           |
| Tail ... ..               | 28                       | 5                           | 17                           |
| Rectum ... ..             | 30                       | 0                           |                              |
| Vagina ... ..             | 4                        | 0                           |                              |
| <i>B. Cowshed</i>         |                          |                             |                              |
| Air (samples) ... ..      | 8                        | 7                           | 85                           |
| Floor ... ..              | 13                       | 10                          | 77                           |
| Faeces on floor ... ..    | 36                       | 5                           | 14                           |
| Cleansing utensils ... .. | 6                        | 2                           | (33)                         |
| Hose pipe ... ..          | 2                        | 2                           | (100)                        |
| Metal manger ... ..       | 2                        | 0                           |                              |
| Wall ... ..               | 1                        | 0                           |                              |
| Doors ... ..              | 3                        | 0                           |                              |
| Straw ... ..              | 1                        | 0                           |                              |
| Hay ... ..                | 1                        | 0                           |                              |
| Manure ... ..             | 2                        | 1                           | (50)                         |
| Roof ... ..               | 1                        | 0                           |                              |
| Milking stools ... ..     | 3                        | 3                           | (100)                        |
| Milking pails ... ..      | 2                        | 1                           | (50)                         |
| <i>C. Workers</i>         |                          |                             |                              |
| Milkers' boots ... ..     | 5                        | 4                           | (80)                         |
| Milkers' clothes ... ..   | 3                        | 0                           |                              |

It is worthy of note that after treatment of all these cows and disinfection of cows and cowshed at a later date (Stuart & Lancaster, 1949) thorough examination by the same methods showed no *Str. agalactiae*.

The next series of observations were made in 13 farm herds. The results from the first nine herds have already been reported as part of an article on the eradication of *Str. agalactiae* (Stableforth *et al.*, 1949). The findings in the whole 13 herds may be summarised by stating that *Str. agalactiae* was obtained from the teats of 185/489 cows (38 per cent.), from the skin of the body of 24/489 (5 per cent.), from 19/48 pairs of milkers' hands (39 per cent.), from the clothes of 8/39 (20 per cent.) and from 22 per cent. of all other cowshed tests, including the air 12/40 (30 per cent.), floors 14/71 (20 per cent.), cleaning utensils 5/37 (17 per cent.) and milking stools 9/12 (75 per cent.).

#### THE ASSOCIATION OF SORE TEATS WITH *Str. agalactiae* INFECTION

In two infected herds a comparison was made of the carrier rate in cows with and without sores or abrasions, the examination of the swabs being carried out as previously described. The results are given in Table II, from which it will be seen that *Str. agalactiae* could be isolated much more frequently from cows with sores or abrasions than from those with none.

TABLE II

RECOVERY OF *Str. agalactiae* FROM THE TEATS OF COWS WITH AND WITHOUT SORES OR ABRASIONS

| Date    | No. of cows examined | No. of cows with infected teats | Farm A |                           |              |    |     |              | Cows' teats with sores |     |              |
|---------|----------------------|---------------------------------|--------|---------------------------|--------------|----|-----|--------------|------------------------|-----|--------------|
|         |                      |                                 | % +ve  | Cows' teats without sores |              |    |     |              |                        | No. | No. infected |
|         |                      |                                 |        | No.                       | No. infected | %  | No. | No. infected | %                      |     |              |
| 23.6.48 | 31                   | 13                              | 42     | 24                        | 7            | 29 | 7   | 6            | 86                     |     |              |
| 2.7.48  |                      |                                 |        |                           |              |    | 7   | 6            | 86                     |     |              |
| 6.7.48  | 31                   | 13                              | 42     | 25                        | 7            | 28 | 6   | 6            | 100                    |     |              |
| 13.7.48 | 30                   | 9                               | 28     | 26                        | 5            | 20 | 4   | 4            | 100                    |     |              |
| 21.7.48 | 30                   | 8                               | 26     | 27                        | 7            | 26 | 3   | 1            | 33                     |     |              |
| 27.7.48 | 30                   | 6                               | 20     | 27                        | 3            | 11 | 3   | 3            | 100                    |     |              |
| 3.8.48  | 30                   | 0                               |        | 28                        | 0            |    | 2   | 0            |                        |     |              |
| 10.8.48 | 30                   | 6                               | 20     | 28                        | 4            | 14 | 2   | 2            | 100                    |     |              |
| 16.8.48 | 31                   | 0                               |        | 39                        | 0            |    | 1   | 0            |                        |     |              |
| 8.9.48  | 31                   | 7                               | 22     | 29                        | 5            | 18 | 2   | 2            | 100                    |     |              |
| Total   | 274                  | 62                              | 24     | 246                       | 38           | 15 | 37  | 30           | 81                     |     |              |
| Farm B  |                      |                                 |        |                           |              |    |     |              |                        |     |              |
| 29.6.48 | 26                   | 15                              | 58     | 16                        | 5            | 31 | 10  | 10           | 100                    |     |              |
| 12.7.48 | 27                   | 16                              | 59     | 20                        | 10           | 30 | 7   | 6            | 86                     |     |              |
| 15.7.48 | 17                   | 1                               | 6      | 11                        | 1            | 9  | 6   | 0            |                        |     |              |
| 22.7.48 | 26                   | 0                               |        | 23                        | 0            |    | 3   | 0            |                        |     |              |
| 9.8.48  | 27                   | 2                               | 7      | 27                        | 2            | 7  | 0   | 0            |                        |     |              |
| 21.9.48 | 25                   | 3                               | 12     | 25                        | 3            | 12 | 0   | 0            |                        |     |              |
| Total   | 148                  | 37                              | 25     | 122                       | 21           | 17 | 26  | 16           | 61                     |     |              |

In this experiment treatment with C.T.A.B. cream was instituted. Details will be published later.

THE SURVIVAL OF *Str. agalactiae* ON VARIOUS MATERIALS  
CONTAINED IN TUBES DURING SUMMER TIME (MAY TO JULY) AND  
WINTER TIME (NOVEMBER TO FEBRUARY)

Various materials, normally found in the cowshed, were cut or broken into small pieces and placed in glass tubes, each material in a separate series of tubes. A number of the tubes were plugged with cotton-wool and autoclaved at 20 lb. for 30 minutes, the remaining tubes being left open and not sterilised. The material in the tubes was then soaked with a *Str. agalactiae* broth culture or with naturally infected milk for several hours and the excess culture withdrawn. The same strain of test organism was used throughout. The approximate number of *Str. agalactiae* inoculated into the tubes was estimated by plate count. The tubes were kept at various temperatures and examined at short intervals until several negative results had been obtained.

In the summer experiment small pieces of material were transferred with sterile precautions into a tube of udder broth and after incubating for 20 hours at 37° C., a loopful of the top liquid was streaked on a plain blood agar plate. The plates were read after 48 hours' incubation at 37° C. Details and results are shown in Table III.

TABLE III

DAYS OF SURVIVAL DURING MAY, JUNE AND JULY

(All materials were previously autoclaved in cotton wool plugged tubes and soaked with diluted broth culture containing 4,000 viable *Str. agalactiae* per ml.)

| Material          | Time of survival (in days) |           |         |           |
|-------------------|----------------------------|-----------|---------|-----------|
|                   | Cowshed                    | Laborat.  | Refrig. | Incubator |
|                   | 20°-24° C.                 | 20-26° C. | 8° C.   | 37° C.    |
| Dung ... ..       | 60                         | 60        | 27      | 0         |
| Soil ... ..       | 43                         | 38        | 8       | 8         |
| Woollen cloth ... | 38                         | 25        | 38      | 2         |
| Linen cloth ...   | 30                         | 32        | 32      | 2         |
| Hair ... ..       | 29                         | 46        | 24      | 11        |
| Straw ... ..      | 10                         | 0         | 18      | 0         |
| Rubber ... ..     | 0                          | 2         | 8       | 0         |
| Wood ... ..       | 0                          | 2         | 0       | 0         |
| Glass balls ...   | 0                          | 0         | 0       | 0         |
| Broth ... ..      | 46                         | 46        | 27      | 7         |

In the experiment carried out in winter-time, seven series of tubes containing various materials were prepared. Details are shown in Table IV. The plugged and sterilised tubes were examined as in the experiment carried out in the summer-time, except that the udder-broth subculture was plated on selective solid medium as well as on plain blood nutrient agar. From the open non-sterilised tubes separate pieces of material were transferred into tubes of sterile milk and liquid selective medium (see above). These were incubated for

20 hours at 37° C. and one loopful of cream streaked on the surface of a plain blood agar plate and on the surface of the selective solid medium. The plates were incubated for 48 hours at 37° C. If any of the subcultures yielded *Str. agalactiae* the result was recorded as positive.

TABLE IV  
DAYS OF SURVIVAL DURING NOVEMBER, DECEMBER, JANUARY AND FEBRUARY

| Cowshed 3—15° C.  |                           |      |                                   |      |                           | Labora-<br>tory | Incubator | Refrig. |
|-------------------|---------------------------|------|-----------------------------------|------|---------------------------|-----------------|-----------|---------|
| Material          | Sterilised and<br>plugged |      | Non-sterilised and<br>not plugged |      | Sterilised and<br>plugged |                 |           |         |
|                   | N.I.M.                    | B.C. | N.I.M.                            | B.C. | N.I.M.                    | N.I.M.          | N.I.M.    |         |
| Dung ...          | 15                        | 24   | 13                                | 23   | 17                        | 3               | 105*      |         |
| Soil ...          | 21                        | 24   | 13                                | 17   | 14                        | 8               | 105*      |         |
| Woollen cloth ... | 21                        | 8    | 15                                | 14   | 4                         | 2               | 105*      |         |
| Linen cloth ...   | 30                        | 4    | 13                                | 14   | 33                        | 2               | 20        |         |
| Hair ...          | 30                        | 24   | 13                                | 14   | 17                        | 3               | 105*      |         |
| Straw ...         | 21                        | 14   | 15                                | 14   | 33                        | 3               | 28        |         |
| Rubber ...        | 3                         | 3    | 13                                | 14   | 8                         | 3               | 17        |         |
| Wood ...          | 21                        | 1    | 15                                | 14   | 11                        | 3               | 105*      |         |
| Glass balls ...   | 21                        | 4    | 21                                | 14   | 18                        | 2               | 8         |         |
| Leather ...       | 3                         | 1    | 15                                | 14   | 4                         | 1               | 2         |         |
| Paper ...         | 9                         | 11   | 13                                | 14   | 4                         | 3               | 3         |         |
| Cork ...          | 21                        | 3    | 13                                | 14   | 17                        | 3               | 20        |         |
| Metal ...         | 2                         | 8    | 13                                | 14   | 4                         | 3               | 2         |         |
| Lime ...          | 21                        | 11   | 13                                | 14   | 17                        | 3               | 20        |         |
| Brick ...         | 41                        | 24   | 15                                | 17   | 33                        | 3               | 105*      |         |
| Concrete ...      | 2                         | 1    | 2                                 | 1    | 1                         | 2               | 0         |         |
| Milk broth ...    | 41                        | 1    | 21                                | 14   | 9                         | 2               | 20        |         |
| Meat broth ...    | 41                        | 43   | 21                                | 14   | 20                        | 26              | 28        |         |
| Glucose broth ... | 21                        | 1    | 13                                | 14   | 4                         | 2               | 28        |         |

Key : N.I.M. means naturally infected milk containing two million *Str. agalactiae* and 80,000 haemolytic staphylococci per ml.

B.C. Means broth culture of *Str. agalactiae*.

Tests marked \* were discontinued at the 105th day because no further material remained in the tubes.

The results of these two experiments show that, when non-sterilised materials of various kinds were soaked with naturally infected secretion and kept in open tubes in the cowshed at 3° C. to 15° C, survival for two weeks was usual. The results with sterile materials in closed tubes were much more irregular, but showed that considerably longer periods of survival can occur under favourable conditions.

#### THE SURVIVAL OF *Str. agalactiae* ON VARIOUS ARTIFICIALLY INFECTED MATERIALS KEPT IN AN INFECTED COWSHED

Various parts of a cowshed, materials, utensils and clothes which would normally be exposed to contamination with *Str. agalactiae* were examined and shown to be free from this organism. These

sites were artificially infected with *Str. agalactiae* naturally-infected milk. Daily examination of the areas was carried out, until two to three negative tests had been obtained, according to the technique previously described.

TABLE V  
SURVIVAL OF *Str. agalactiae* ON MATERIAL IN  
COWSHED

| <i>Material</i>         | <i>Days of survival</i> |
|-------------------------|-------------------------|
| Floor ... ..            | 4                       |
| Window ... ..           | 5                       |
| Cement wall ... ..      | 10                      |
| Lime wall ... ..        | 7                       |
| Wooden partition ... .. | 1                       |
| Broom handle ... ..     | 5                       |
| Milking stool ... ..    | 1                       |
| Bucket ... ..           | 8                       |
| Straw ... ..            | 13                      |
| Hay ... ..              | 20                      |
| Uddercloth ... ..       | 7                       |
| Linen coat ... ..       | 2                       |
| Rubber coat ... ..      | 2                       |
| Rubber boots ... ..     | 4                       |
| Paper box ... ..        | 4                       |
| Iron manger ... ..      | 4                       |

The results are shown in Table V; they indicate that of the materials examined *Str. agalactiae* survived longest on hay (20 days). The survival time on the bucket was eight days, on the wall ten or seven days, on the udder cloth seven days, window ledge and broom five days. The shorter period of survival as compared with the previous experiments may possibly have been due to the greater mechanical difficulty of recovering material from the infected sites.

The areas of the wall used for these observations were then sprayed with 5 per cent. aqueous solution of cresol. Two days later *Str. agalactiae* was implanted on this area and subsequent examinations showed that this organism did not survive 24 hours. When *Str. agalactiae* was again implanted on this area 14 days' later, subsequent examinations showed that the organism survived only two days.

#### THE SURVIVAL OF *Str. agalactiae* ON VARIOUS AREAS OF THE SKIN OF FOUR COWS

Selected areas of the skin of three cows and of a heifer previously found to be free from *Str. agalactiae* were smeared with broth or milk culture of this organism or with naturally infected milk, once or twice daily for three days in order to implant the organisms on the skin. Swabs were then taken every day from these areas until several negative tests were obtained. The method of recovery of *Str. agalactiae* was similar to that used in the previous experiments.

TABLE VI  
THE MAXIMUM SURVIVAL OF *Str. agalactiae* ON THE SKIN OF THREE COWS AND A HEIFER

| Age of animal             | 8                                                                                                              | 10                | 6                      | 3½                                      |
|---------------------------|----------------------------------------------------------------------------------------------------------------|-------------------|------------------------|-----------------------------------------|
| Area infected             | Duration of infection (in days)                                                                                |                   |                        |                                         |
| Cow                       | (Ayrshire) Cn.                                                                                                 |                   |                        |                                         |
| Month                     | Feb., March, April, May                                                                                        | March, April, May | March, April, May      | Guernsey heifer II<br>March, April, May |
|                           | N.I.M.<br>B.C. OR A.I.M. B.C. A.I.M. B.C. A.I.M. B.C. A.I.M. B.C. A.I.M. A.I.M. B.C. N.I.M. B.C. A.I.M. A.I.M. |                   |                        |                                         |
|                           | S.B.C.                                                                                                         |                   | N.I.M.<br>OR<br>S.R.C. |                                         |
| Teats                     | 0 3 3 2                                                                                                        | 2 2 3 3           | 1 2 0 0                | 0 0 0 0                                 |
| Udder                     | 4 3 0                                                                                                          | 0 2 0 3           | 4 1 0 0                | 0 0 0 0                                 |
| Mouth                     |                                                                                                                |                   |                        |                                         |
| Skin over Masseter muscle |                                                                                                                |                   | 1                      |                                         |
| Horn (base)               | 26                                                                                                             | 6                 | 2 3 2                  | 5 0 8 7                                 |
| Ear                       |                                                                                                                |                   |                        |                                         |
| Neck—Unshaved             | 4 11                                                                                                           |                   | 7 4                    | 8                                       |
| Shaved                    | 3 22                                                                                                           | 6 8 7             | 0 0                    | 0 6                                     |
| Thigh—Unshaved            | 19                                                                                                             |                   |                        |                                         |
| Shaved                    |                                                                                                                | 3 10 0            | 2 0                    | 0 8                                     |
| Flank—Unshaved            | 7                                                                                                              |                   |                        |                                         |
| Shaved                    | 6 6                                                                                                            | 9                 | 3 2 8 6 0 4            | 3 0 5 4                                 |
| Legs                      |                                                                                                                |                   | 7                      |                                         |
| Feet                      |                                                                                                                |                   |                        |                                         |
| Tail                      | 23                                                                                                             | 2                 | 3 4 3 0                | 1 4 0 4                                 |
|                           |                                                                                                                |                   |                        | 5 0 4 5                                 |

B.C. = Broth culture, A.I.M. = Artificially infected milk, N.I.M. = Naturally infected milk, S.B.C. = Serum broth culture.

Table VI shows considerable variation from cow to cow. On the skin of cow Lo, one of two older cows, the organisms survived for a longer period (up to 26 days) than on that of the younger cow G.I. or on the heifer G.II, where the maximum time of survival was eight days. From some areas of cows' skin, *e.g.*, head (base of horn), tail (base) and neck *Str. agalactiae* was recovered for three weeks following infection, while on the teats and on the udder *Str. agalactiae* survived for only three to four days.

#### DISCUSSION

The results described have confirmed previous observations regarding the occurrence of *Str. agalactiae* in an infected herd in various sites other than the milk. With the help of the method and medium described it has also been possible to show that it is much more widespread than previously believed. This observation, together with the demonstration that *Str. agalactiae* may survive for two weeks and possibly much longer (selective methods can never give 100 per cent. recovery from highly contaminated materials and mechanical difficulties are also involved in cowshed work), suggests that infection of cows may occur from sources other than milk and that disinfection of the cow's skin and her environment is likely to aid considerably in eradication of the infection from the herds. The much higher incidence of infection found on the teats showing sores as compared with those from which they were absent suggests also the importance of attention to sores and abrasions and the need, when choosing disinfectants for cowshed use, of avoiding any which are likely to cause damage to the skin. The importance of sore teats as sources of infection is also emphasised by the fact, frequently observed but not dealt with in this paper, that, by impression cultures, *Str. agalactiae* can often be cultivated from such sores in large numbers.

#### SUMMARY

Using methods already described, the incidence of *Str. agalactiae* in various sites other than milk has been determined.

Observations in a highly infected laboratory herd showed a widespread distribution of the organism on the outside of the cow's body, and parts of the cowshed and utensils.

Observations in 13 farm herds showed that *Str. agalactiae* was recovered from 38 per cent. of teat examinations, 5 per cent. of skin examinations, 38 per cent. milkers' hands (pairs), 20 per cent. of clothes and 22 per cent. of tests of air, utensils, floors, etc.

In two herds, *Str. agalactiae* was obtained from 61 and 81 per cent. of teats with sores and from 15 and 17 per cent. of those without sores.

Various materials or sites artificially infected in the cowshed remained positive for one to 20 days. Unsterilised materials (dung, hair, wood, cloth, brick, etc.) soaked with *Str. agalactiae* milk cultures kept at cowshed temperatures in summer and winter, usually re-

mained positive for about two weeks. Sterilised materials were positive up to two months showing that *Str. agalactiae* could survive for this period under favourable conditions.

*Str. agalactiae* in milk or culture smeared on various parts of the cows' skin could be recovered for one to 26 days.

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THE ENDOTOXIN OF *BACT. COLI*

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## INTRODUCTION

NUMEROUS studies concerned mainly with the more prominent members of the salmonella and dysentery groups have shown that, with the probable exception of *Sh. shigae*, the toxicity and other biological activities of bacteria of the colon-typhoid-dysentery group are attributable to a nitrogen containing polysaccharide complex. The toxicity reported in *Bact. coli* cultures by some early authors was probably due to this component (Vincent, 1925; Steinberg and Ecker, 1926; Smith and Little, 1927). This complex, referred to by some authors as somatic antigen and by others as endotoxin, was extracted from bacterial bodies with trichloroacetic acid by Boivin and Mesrobian (1933), by tryptic digestion by Raistrick and Topley (1934) and with diethylene glycol by Morgan (1937). It is antigenic (Raistrick and Topley, 1934), it gives rise to a pathological picture in which the fundamental change is a terminal hypertension in the circulatory system (Delaunay *et al.*, 1947), it causes hyperthermia (Co Tui *et al.*, 1944), a transient hyperglycaemia followed by a hypoglycaemia (Delafield, 1934), leucopaenia (Dennis and Senekjian, 1939), haemorrhage in the placenta with abortion, and haemorrhage in implanted tumours (Zahl and Hutner, 1943; Zahl and Bjerknes, 1943), and its toxicity appears to be less in animals injected with adrenal cortical extract (Lewis *et al.*, 1946). There is no evidence in the literature that endotoxins from different species of Gram-negative bacteria give different biological reactions, although, for the purposes of this investigation, it was assumed that the endotoxin of *Bact. coli* might differ from those of salmonella and dysentery organisms. The question of the specificity of immunity to endotoxin among members of the group is undecided, as shown by Zahl and Hutner (1944).

While endeavouring to explain the toxicity of some heat-killed saline suspensions of *Bact. coli* in guinea-pigs, it was found that the toxic substance was a polysaccharide complex, or endotoxin, and that it was present in filtrates of heated suspensions. These filtrates were not toxic for mice within the limits imposed by the volume of the injection. High susceptibility to endotoxin in guinea-pigs and the presence of endotoxin in the filtrate of heated saline suspensions appeared to conflict with accepted knowledge and because of this the investigation reported here was undertaken. When it had become clear that the endotoxin of *Bact. coli* was liberated into the suspending fluid under the influence of heat, the researches were continued to demonstrate that it could elicit the main biological reactions and had the properties usually associated with the

endotoxins of Gram-negative bacteria. It appeared to be important to determine whether the phenomena observed occurred with other organisms and therefore some of the experimental work was repeated with salmonella and dysentery cultures. Work on dose-mortality relation and toxicity was carried out in an effort to explain why the release of endotoxin by heat could be observed with *Bact. coli* using guinea-pigs, when it had been overlooked in so many studies on other organisms when mice had been used.

A method for the preparation of the polysaccharide complex from filtrates of heated synthetic medium cultures was developed, and a technique for mass cultivation of bacteria, which may contain some new features, was used.

#### MATERIALS

*Strains.*—The *Bact. coli* strains used were from pathological conditions in calves. One of these, selected almost at random, was used for all experimental work. The salmonella and dysentery strains were laboratory strains.

*Media.*—Digest broth was a papain digest of horse muscle adjusted to 2 per cent. of solids with 0.5 per cent. of glucose added. Asparagine medium was a modification of that commonly used in the preparation of tuberculin, and contained 0.4 per cent. asparagine, 0.6 per cent. sodium citrate, 0.2 per cent. of disodium phosphate, 0.1 per cent. of ammonium chloride, 0.05 per cent. of magnesium sulphate, 0.005 per cent. of iron ammonium citrate, and 5 per cent. of glycerine or glucose. In order to reduce the cost of this medium, in most experiments the asparagine was replaced with a five-day acid hydrolysate of horse muscle and this medium is referred to as hydrolysate medium; the amount of hydrolysate was regulated to give the same nitrogen content as in the asparagine medium. Towards the end of the investigation the asparagine was replaced with an equivalent weight of ammonium sulphate, and this medium is referred to as ammonium sulphate medium. The ammonia medium used by Johnstone (1942) is referred to as the "conventional ammonia medium"; this consisted of ammonium sulphate 0.05 per cent.; potassium dihydrogen phosphate 0.025 per cent.; magnesium sulphate 0.002 per cent. and glucose 0.125 per cent. Glucose agar was prepared from digest broth and asparagine agar from asparagine broth.

*Experimental Animals.*—Guinea-pigs were obtained from a number of independent units, and were of mixed breeds. The mean weight within groups was 350 gm., and the sexes were in equal numbers, or nearly so. In comparative experiments groups were made homogenous by matching each guinea-pig in one group by sex and weight with a corresponding guinea-pig in the other groups. Environmental temperature was maintained at 65° C. and was recorded. Mice were 18 to 22 gm. and, except when varied by design, were from one breeding unit.

#### METHODS

*Identification of cultures.*—Strains of *Bact. coli* were identified by the cultural and biochemical criteria of Kauffman (1947). Aerated cultures were examined in smears, by flooded and streak cultures on glucose agar plates, on McConkie plates and additionally in the case of salmonella and dysentery cultures, by agglutination with standard antisera.

*Cell Counts.*—Viable counts were obtained by plating 0.2 ml. of the same culture dilution on each of eight glucose agar plates, aiming at two-figure

counts and standard deviations of less than 20 per cent. Opacity estimates were made with a Spekker absorptiometer using a 1 cm. cell and a neutral filter, and taking 0.40 to correspond to 750 million cells.

*Air Sterilisation.*—The air was passed in series through sulphuric acid, a 3 ft.  $\times$  2½ in. column of granulated charcoal and a 6 in.  $\times$  2 in. slag wool filter. The charcoal and slag wool were sterilised by autoclaving and dried in a hot air oven. The procedure was controlled by periodic tests with uninoculated broth.

*Filtration.*—Cultures and saline suspensions were clarified by centrifuging or with clarifying pads and filtered through Ford's S.B. pads. Filtrates not derived from heated suspensions were proved sterile and then heated at 80° C. for one hour.

*Animal Inoculation.*—Mouse injections were made in 0.2 ml. and guinea-pig injections in 1.0 ml. amounts except where otherwise stated.

*Calculation of  $LD_{50}$ .*—The method of Reed and Muench (1938) was employed in mouse experiments and, when possible, in guinea-pig experiments. In other guinea-pig experiments, estimates were obtained by taking a reading or the average of readings from a standard line.

*Preparation of Dense Cultures by Aeration.*—The success reported by Heatley and Florey (1946) in the preparation of *Bact. coli* antibiotic by aeration suggested its use in this investigation, and preliminary experiments demonstrated that cultures with 25,000 million viable cells per ml. could be obtained. The principles involved appeared to be that the aeration should be of such vigour as to maintain all the culture fluid in an agitated condition unceasingly and that the nutritive constituents of the medium should be present in large amounts, with an approximate balance between the sources of nitrogen and carbon as judged by residual carbohydrate at the end of growth. Such a balance appeared to be given by 0.6 per cent. of ammonium sulphate and 3.0 per cent. of glucose, but the media used in this investigation contained a higher proportion of carbohydrate. There is no reason to suppose that all the salts used were necessary. The medium was incubated in 10-litre quantities in 20-litre containers in a water bath. An inoculum of 200 ml. of incubator grown culture in the same medium was employed and the culture was grown for 9 to 13 hours. Throughout this time air was delivered into the medium through one or two Aerox tubes, 2 in.  $\times$  3 in., grade 28, at about 0.75 cu. ft. per minute. Foam was not a serious problem in the biuret negative media which were generally used, but it increased as growth approached its maximum. It could be controlled with small amounts of cetyl alcohol, but this was not used in any experimental culture. When peptone broth was used foam was a serious hindrance and was controlled with partial success with 0.1 per cent. of lard. In general, the viable cell counts observed in cultures prepared by this method were 20,000 million organisms per ml. in hydrolysate or asparagine media, 10,000 million in ammonium sulphate medium and 3,000 million in peptone broth; no attempt was made to improve the latter by adjustment of the constituents and it was seldom used except for the cultivation of *Sh. shigae*.

The liquid cultures used in this investigation were prepared by this method and, except when specified otherwise, they were in hydrolysate, asparagine or ammonium sulphate medium. Suspensions prepared from agar growths are specifically referred to as "saline suspensions".

## EXPERIMENTAL RESULTS

*The Release of Endotoxin from the Cells*

Twenty-one strains of *Bact. coli* derived from pathological conditions in calves were heated at 80° C. for one hour, and were injected into guinea-pigs in doses of 100,000 million organisms in 5 ml. Sixteen strains killed 5/5 guinea-pigs, four strains killed 4/5 and one 3/5. In measuring this toxicity the data available suggested that a single level test and a standard line would best meet the two problems of exercising the maximum economy in the use of guinea-pigs and of obtaining results in a comparable form. The toxic substance was abundant in filtrates of heated saline suspensions and these could be concentrated *in vacuo*. Six groups of 15 guinea-pigs were injected with such a concentrate, with dose intervals of two, and from the results the line shown in Fig. 1 was drawn. As shown in this figure, the direction of this line was confirmed later by experiments on endotoxin in the filtrate of unheated culture and on endotoxin complex. At the end of the investigation the direction of the line was further examined by assembling the results of all experiments other than those used in Fig. 1, in which two groups of 20 guinea-pigs had been injected simultaneously with endotoxin (Second section of Table I). When the dose (column "b") that was observed to give the mortalities in column "d" was taken as 100, the dose that should have given these mortalities according to the slope (column "f"), was 87. Thus, readings taken from the slope according to these figures exaggerated slightly the difference in mortality resulting from different dosage; in brief, the slope of the standard line was too steep. This discrepancy did not appear to be of sufficient importance to warrant correction of results.

Filtrates from suspensions which had been heated at 80° C. for one hour were almost as toxic as the unfiltered suspensions themselves. Thus, batch 16, a culture in ammonium sulphate medium, after heating at 80° C. for one hour, gave mortalities of 8/20 in guinea-pigs in each of two replicates in doses of 2 ml., a  $LD_{50}$  of 2.6 ml. A filtrate of this heated culture, when injected in doses of 1.0 ml. and 4.0 ml. gave mortalities of 7/20 and 10/20, a  $LD_{50}$  of 2.7 ml., while the organisms in terms of equivalents of the original culture, gave mortalities of 0/20 and 6/20 in doses of 2.0 ml. and 8.0 ml., a  $LD_{50}$  of 12.8 ml. or more. In this experiment it would thus appear that about 80 per cent. of the endotoxin was in the filtrate.

It was found that endotoxin could be detected in filtrates of unheated suspensions if large doses were injected in groups of 20 guinea-pigs. The critical question in the investigation was, therefore, whether the toxicity of the suspending fluid did in fact increase when the suspension was heated, and with this in mind a series of experiments were carried out. In each experiment a sample of freshly grown culture or saline suspension was adjusted

TABLE I  
RELATION BETWEEN A DOSE ARRIVED AT BY EXPERIMENT AND CALCULATION,  
USING PAIRED GROUPS OF 20 GUINEA-PIGS

| <i>Ratio of<br/>doses used</i> | <i>Mortality<br/>observed</i> | <i>Calculated ratio<br/>to give the same<br/>mortality</i> | <i>Dose f as<br/>percentage<br/>of dose b</i> |
|--------------------------------|-------------------------------|------------------------------------------------------------|-----------------------------------------------|
| <i>a. b.</i>                   | <i>c. d.</i>                  | <i>e. f.</i>                                               |                                               |
| 1:1                            | 15 and 15                     | 1:1.0                                                      | 100                                           |
| 1:1                            | 9 " 11                        | 1:1.2                                                      | 120                                           |
| 1:1                            | 3 " 4                         | 1:1.2                                                      | 120                                           |
| 1:1                            | 11 " 11                       | 1:1.0                                                      | 100                                           |
| 1:1                            | 11 " 14                       | 1:1.4                                                      | 140                                           |
| 1:1                            | 11 " 14                       | 1:1.4                                                      | 140                                           |
| 1:1                            | 7 " 6                         | 1:1.1                                                      | 110                                           |
| 1:1                            | 1 " 2                         | 1:1.4                                                      | 140                                           |
| 1:1                            | 1 " 1                         | 1:1.0                                                      | 100                                           |
| 1:1                            | 1 " 2                         | 1:1.4                                                      | 140                                           |
| 1:1                            | 1 " 2                         | 1:1.4                                                      | 140                                           |
| 1:1                            | 2 " 2                         | 1:1.0                                                      | 100                                           |
| 1:1                            | 1 " 2                         | 1:1.4                                                      | 140                                           |
| 1:1                            | 8 " 8                         | 1:1.0                                                      | 100                                           |
| 1:2                            | 18 " 19                       | 1:1.4                                                      | 70                                            |
| 1:2                            | 8 " 10                        | 1:1.3                                                      | 65                                            |
| 1:2                            | 11 " 15                       | 1:1.7                                                      | 85                                            |
| 1:2                            | 11 " 15                       | 1:1.7                                                      | 85                                            |
| 1:2                            | 11 " 11                       | 1:1.0                                                      | 50                                            |
| 1:2                            | 9 " 11                        | 1:1.2                                                      | 60                                            |
| 1:2                            | 6 " 10                        | 1:1.6                                                      | 80                                            |
| 1:2                            | 2 " 4                         | 1:1.5                                                      | 75                                            |
| 1:2                            | 6 " 10                        | 1:1.6                                                      | 80                                            |
| 1:2                            | 1 " 6                         | 1:2.9                                                      | 145                                           |
| 1:2                            | 11 " 14                       | 1:1.4                                                      | 70                                            |
| 1:2                            | 1 " 5                         | 1:2.4                                                      | 120                                           |
| 1:2                            | 13 " 18                       | 1:2.4                                                      | 120                                           |
| 1:4                            | 11 " 15                       | 1:1.7                                                      | 42                                            |
| 1:4                            | 9 " 15                        | 1:2.1                                                      | 53                                            |
| 1:4                            | 11 " 15                       | 1:1.7                                                      | 42                                            |
| 1:4                            | 9 " 15                        | 1:2.1                                                      | 53                                            |
| 1:4                            | 4 " 18                        | 1:7.3                                                      | 183                                           |
| 1:4                            | 3 " 18                        | 1:8.6                                                      | 215                                           |
| 1:4                            | 1 " 10                        | 1:4.7                                                      | 117                                           |
| 1:4                            | 7 " 10                        | 1:1.4                                                      | 35                                            |
| 1:10                           | 3 " 18                        | 1:8.6                                                      | 86                                            |
| 1:20                           | 3 " 19                        | 1:12.2                                                     | 61                                            |
| Mean                           |                               |                                                            | 87                                            |

to pH 7.0 and was divided into two equal parts. One part was filtered and the filtrate was heated at 80° C. for one hour. The other half was heated at 80° C. for one hour and then filtered. The results of 10 of these experiments are shown in Table II. It will be seen that there was a sixfold average increase in the toxicity of the filtrates due to the heating of the suspension or culture; or, alternatively, that about one-sixth of the endotoxin was already in the suspending fluid before the heating. Additional experiments were performed to ascertain whether the phenomenon was other

than a simple release of endotoxin. For example, the  $LD_{50}$  of a filtrate of heated culture, Batch 61, was 0.9 ml. When the unheated cells were washed twice and then heated as a saline suspension the filtrate killed 9/20 guinea-pigs in a dose of 2.0 ml., a  $LD_{50}$  of 2.2 ml., thus showing that the phenomenon was not dependent on any factor present in the filtrate. The  $LD_{50}$  of the filtrate of a heated culture, Batch 87, was 0.65 ml., but when the organisms from the heated culture were resuspended in saline solution and again heated at 80° C. for one hour, no toxin could be detected in the supernatant, the filtrate in doses of 5 ml. being innocuous in 20 guinea-pigs. This absence of any substantial residue of endotoxin in the cells after heating was further confirmed by failure to extract any alcohol precipitable matter by the diethylene glycol technique of Morgan (1937). Endotoxin was lost when cells were washed at room temperature; the filtrate of heated culture, Batch 65, killed 8/20 guinea-pigs in a dose of 5 ml., a  $LD_{50}$  of 6.5 ml., but when the living cells were washed three times, resuspended in saline and heated the filtrate of the heated suspension killed only 3/20 guinea-pigs in a dose of 10 ml., a  $LD_{50}$  of 26 ml. It will be seen that this had occurred, but to a less extent, in the experiment on Batch 61 above. The duration and temperature of the heating were not critical factors. For example, a culture was divided into two parts and one part was heated for one hour at 80° C. and the other at 60° C. The filtrate of the former killed 10/20 guinea-pigs and that from the latter 9/20 guinea-pigs. These experiments have thus failed to reveal any phenomenon which is not in accord with the hypothesis that the endotoxin is washed out of the cells by water and that the process is complete, or almost complete, after one hour at 80° C.

TABLE II  
RELATIVE TOXICITIES OF THE FILTRATES OF HEATED AND UNHEATED  
SUSPENSIONS OF *Bact. coli*.

| Batch | Filtrate of |            | Filtrate of |            | $LD_{50}$ |        |
|-------|-------------|------------|-------------|------------|-----------|--------|
|       | unheated    | suspension | heated      | suspension | Unheated  | Heated |
|       | Doses       | Deaths     | Doses       | Deaths     |           |        |
|       | ml.         |            | ml.         |            | ml.       | ml.    |
| 52    | 2.0         | 0/20       | 2.5         | 7/20       | 10.0      | 3.5    |
|       | 5.0         | 4/20       |             |            |           |        |
| 56    | 7.5         | 1/20       | 4.0         | 36/60      | 35.0      | 3.1    |
| 61    | 5.0         | 7/20       | 0.5         | 6/20       | 7.0       | 0.9    |
|       |             |            | 1.0         | 10/20      |           |        |
| 63    | 5.0         | 3/20       | 0.5         | 2/20       | 13.0      | 1.9    |
|       |             |            | 1.0         | 4/20       |           |        |
| 65    | 10.0        | 1/20       | 5.0         | 8/20       | 47.0      | 6.5    |
| 75    | 10.0        | 3/20       | 5.0         | 8/20       | 26.0      | 6.5    |
| 87    | 5.0         | 11/20      | 0.5         | 8/20       | 4.5       | 0.6    |
| 89    | 5.0         | 15/20      | 0.5         | 10/20      | 2.6       | 0.5    |
| 93    | 5.0         | 13/20      | 0.5         | 4/20       | 3.1       | 1.1    |
| 96    | 5.0         | 10/20      | 1.0         | 9/20       | 5.0       | 1.1    |
| Mean  |             |            |             |            | 15.32     | 2.57   |

*Preparation of Endotoxin Complex from Filtrate of Heated Suspensions*

The presence of endotoxin in solution in these filtrates unaccompanied by other non-dialysable medium constituents, offered opportunities for its preparation in large quantities. The endotoxin complex could not be isolated directly from the filtrate of heated culture by alcohol precipitation, because of the presence in the filtrate of large quantities of other alcohol precipitable matter, presumed to be amino-acids. The removal of these by dialysis led to dilution, so that the endotoxin complex did not form a visible precipitate; in any case, dialysis at this stage would have been impractical owing to the need to handle large amounts. The method used, therefore, was to concentrate the filtrate under reduced pressure at 60°C. to 1/20 volume, dialyse 16 hours, concentrate to 1/50 of the original volume, dialyse 48 hours against running tap water and a further 24 hours against static distilled water at 4°C., precipitate in 75 per cent. alcohol and resuspend the precipitate in distilled water or dry with ether. Less time was allowed for dialysis in some batches. Since the avoidance of contamination was of paramount importance, primary consideration was not given to yields, but it is probable that they were generally satisfactory. The dry weight of organisms in a well-grown hydrolysate medium was 0.2 to 0.4 g. per 100 ml. The yield of endotoxin from such a culture was about 0.03 gm. per 100 ml. or more, or 10 per cent. of the dry weight of cells, a recovery rate which agrees with that commonly reported (Weil, 1943). The possibility of a gross error in this calculation and in those of other workers due to admixture of debris or precipitates with the cell, must not be overlooked. This method of preparing polysaccharide complex did not provide for the separation of high molecular weight nitrogenous material and was, therefore, not applicable to filtrates of peptone broth cultures. Nevertheless, by taking advantage of the tendency of the high molecular weight material to adhere to glass after alcohol precipitation, the method could give rough purification and concentration and was used in a few batches.

It was demonstrated by experiments in guinea-pigs that in a concentrated dialysed filtrate from an ammonium sulphate culture, the endotoxin was substantially removed from solution by alcohol precipitation and, in the same material, it was shown that 97 per cent. of the solid matter was recovered; alcohol precipitation must be looked upon, therefore, more as a method of recovering endotoxin from solution than as one of purification. These experiments were carried out to eliminate the possibility that the alcohol precipitation of endotoxin from hydrolysate culture filtrate might have depended on the presence of alcohol-precipitable media constituents.

*Physical and Chemical Properties of the Isolated Complex*

The substance obtained by alcohol precipitation was a greyish

white powder, although when the final recovery from dialysed filtrates was accomplished by evaporation to dryness under reduced pressure, an active glass-like substance was obtained. All work reported in this paper was carried out with alcohol precipitated material.

The complex could be dissolved in water at 60° C. and 80° C. with greater ease than at room temperature and 2.5 per cent. solutions could be prepared in a few hours at these higher temperatures. Solutions were markedly fluorescent and, in fact, the presence of endotoxin in unconcentrated filtrates of heated cultures could be detected by this property. It was soluble in phenol, acetic acid, trichloroacetic acid, sulphuric acid, alum and metaphosphoric acid, but was precipitated by phosphotungstic acid. The biuret reaction was negative, except in batches in which biuret+ material had been present in the culture medium.

Several attempts were made to assess the amount of destruction which occurred in one hour at 80° C., but it was not great and, therefore, not easy to demonstrate; it appeared to be about 12 per cent. as suggested by the following experiment:—Groups of 12 mice were injected intraperitoneally with 0.1 ml., 0.25 ml., and 0.025 ml., of a solution of endotoxin, batch 116. The endotoxin solution was heated at 80° C. for one hour and similar groups were injected with the heated solution. Mortality from the unheated solution was 12, 10 and 3, and from the heated solution 11, 7 and 3, giving LD<sub>50</sub> values of 0.037 ml. and 0.042 ml. respectively. There was thus a loss of 12 per cent. after heating. It was later shown, using *S. typhimurium* endotoxin intravenously, that destruction by heat was not affected by the reaction of the solution; while the unheated solution killed 16/20 and 18/20 mice in doses of 0.03 ml. and 0.04 ml, the same doses heated at pH 5.0 killed 10/20 and 16/20, at pH 7, 8/20 and 17/20 and at pH 9, 11/20 and 16/20. A number of batches were examined chemically by Mr. C. A. MacDonald of this Institute and the results are shown in Table III. They show the consistent presence in the complex of about 4 per cent. nitrogen and a yield of 20 to 40 per cent. carbohydrate in terms of glucose after hydrolysing with strong acid for two hours. No particular attempt was made to obtain uniformity of composition in these samples and it is probable that the nitrogen and carbohydrate contents were affected by the composition of the original medium, residual media constituents and efficiency of dialysis. For example, Batch 87, dialysed in three parts, for 24 hours, 48 hours and 72 hours, gave nitrogen percentage of 4.37, 3.86 and 3.50 respectively. Batches 75 and 116 were dialysed for 24 hours and had a high N content, while batch 108 grown in a dilute medium and dialysed for 72 hours contained only 2.54 of nitrogen; in Batch 63 a high N content was related to an excessive amount of N in the culture medium (Table III).

TABLE III  
CARBOHYDRATE, IN TERMS OF GLUCOSE, AFTER HYDROLYSIS AND NITROGEN  
(PERCENTAGES IN DRY WEIGHT OF ENDOTOXIN)

| Batch | Medium                     | Glucose * | Nitrogen |
|-------|----------------------------|-----------|----------|
| 56    | Asparagine                 | 25.12     | 2.73     |
| 57    | Asparagine agar.           | 40.23     | 2.34     |
| 63    | Hydrolysate (Excess N)     | 35.10     | 5.60     |
| 75    | Hydrolysate                | 29.83     | 4.34     |
| 76    | "                          | 21.84     | 3.37     |
| 77    | "                          | 21.77     | 3.90     |
| 93    | "                          | 18.09     | 3.50     |
| 116   | Ammonia                    | 24.30     | 4.80     |
| 103   | Hydrolysate (uninoculated) | 0.75      | 2.61     |

\* Less small amounts present before hydrolysis.

### Biological Reactions

Guinea-pigs injected with a fatal dose of *Bact. coli* endotoxin developed a clearly defined train of symptoms. Depression and restlessness were observed in about one hour, an unsteadiness and characteristic flaccidity and flattening of the abdomen and a progressive increase in the rate of respiration in the ensuing hour leading, in about three hours, to the assumption of a well-defined posture in which the animal rested with its abdomen in a flaccid state on the floor, breathing rapidly, its head on the floor and the two hind limbs with flexed hocks extended backwards; this posture was not abandoned when the animal was prodded. Death, sometimes preceded by convulsive leaps, occurred most commonly eight to ten hours after injection. Smaller doses induced a state of debility in which deaths might occur as long as ten days after the injection. At post-mortem examination fluid in the peritoneal cavity was invariably in excess, but varied in amounts from a generally moist state of the viscera to a removable excess of 20 ml. A limpness of the alimentary tract reflected the abdominal flaccidity. Incidental cutting did not lead to bleeding. A bruised appearance of the stomach was common and a haemorrhagic appearance of the small intestines was observed occasionally. Patches of capillary congestion in the submucosa of the colon gave rise to striking purple splashes, a lesion seen in about 80 per cent. of acute cases. A small proportion of carcasses failed to show any gross lesion other than a general moisture and limpness of viscera and the absence of accidental post-mortem bleeding. Tissues examined microscopically showed accumulation of blood cells with no clear evidence of extravasation, indicating a deployment of blood into peripheral tissues.

Three calves from six to 21 days old were injected subcutaneously with 4 mgm. of endotoxin, Batch 56, or about six guinea-pig lethal doses, and one six-day-old calf was injected intravenously with 0.4 mgm. of the same toxin. Symptoms commenced 40 minutes after

injection and were severe for about six hours; the animals were unable to stand, breathing violently and in extreme distress. Their behaviour corresponded closely to that of calves injected with filtrates of shallow culture of *Bact. coli* as recorded by Smith and Little (1927). A temperature reaction, a precipitous fall in the leucocyte count and a hyperglycaemia followed by spectacular hypoglycaemia occurred over a period of six hours, and corresponded approximately in time with the incidence of gross symptoms (Table IV).

TABLE IV  
REACTIONS TO *Bact. coli* ENDOTOXIN INJECTION IN A CALF  
DOSE: 4.0 MGM. SUBCUTANEOUSLY

| Time in<br>hours after<br>injection | Temperature | Leucocyte<br>count | Blood<br>sugar<br>(mgm./100 ml.) | General symptoms       |
|-------------------------------------|-------------|--------------------|----------------------------------|------------------------|
| 0                                   | 102.8       | 7,600              | 54.5                             | Normal                 |
| 1                                   | 103.0       | 4,900              | 51.5                             | Animal down. Dyspnoea  |
| 2                                   | 104.2       | 1,700              | 76.0                             | Collapsed. Diarrhoea   |
| 3                                   | 102.8       | 1,850              | 78.0                             | " "                    |
| 4                                   | 101.2       | 1,400              | 30.0                             | " "                    |
| 5                                   | 102.0       | 3,900              | 5.3*                             | Respiration slower     |
| 6                                   | 101.8       | 5,300              | 11.8                             | " "                    |
| 7                                   |             |                    |                                  | Recovery from collapse |

\* Approximate.

Twenty-five mice, judged from appearance and from the date of mating to be 15 to 18 days pregnant, were divided into five groups of five. Each of these groups was injected intravenously with 1/10 LD<sub>50</sub> of one sample of the purified endotoxin from Batches 60, 76, 77, 87 and 93. Two of the 25 mice died and the remaining 23 were killed 15 hours after injection. On post-mortem examination, in 21 animals the uteri were haemorrhagic, relaxed and empty, and in the other four displaced foetuses with extensive placental haemorrhages were present.

A group of 20 guinea-pigs was injected intramuscularly with 1.0 ml. of cortical extract and after three hours were injected, together with a control group, with an estimated LD<sub>50</sub> of endotoxin, Batch 56; four treated animals and eleven controls died, thus suggesting some protective effect by the cortical extract.

Deaths occurred readily in guinea-pigs following intradermal injections of purified endotoxin. In those which survived, a blanched weal about 15 mm. in diameter appeared two hours after injection and disappeared by the 18th hour, leaving no evidence of necrosis. Unlike the results reported with *S. typhi* filtrates by Beeson (1947) a skin reaction could not be provoked by intracardiac injection of small doses of coli endotoxin.

In view of the suspicion aroused by the microscopic appearance of the tissues that the permeability of capillaries in guinea-pigs killed with endotoxin was affected, attempts were made to demonstrate a

local effect of this nature. A group of four guinea-pigs was injected intradermally with sufficient purified endotoxin to produce a weal, and half an hour later they were injected intracardially with 2 ml. of 1 per cent. aqueous trypan blue. It was expected that capillary permeability would be revealed by the appearance of a blue ring (Menkin, 1938). No such reaction appeared and the local weal in fact retained a blanched appearance in contrast to the surrounding blue. A biuret positive endotoxin concentrate prepared from growth washed off glucose agar and concentrated by evaporation gave a positive reaction probably attributable to the peptone. The results of these experiments thus supported the view that the essential pathological change was peripheral hypertension rather than permeability.

### *Immunity*

It was recognised that the subject of immunity was a complex one, and no attempt was made to go farther than to show that under some conditions the polysaccharide complex of *Bact. coli* conferred immunity against further injections, and that passive protection against injections of living organisms was conferred. The endotoxin complexes used in the ensuing experiments were prepared from growths in hydrolysate media.

Seventy guinea-pigs were assembled from the survivors of toxin estimation experiments in which deaths had varied from 0/20 to 7/20. Twenty of these, selected proportionately from their groups, and 20 controls were injected with concentrated endotoxin two to four weeks after their first toxin injections. Nineteen, or 95 per cent. of the controls, and 7, or 35 per cent. of the treated animals, died. The remaining 50 treated animals and 20 controls were injected with *S. enteritidis* endotoxin. Twelve, or 24 per cent. of the treated guinea-pigs, and 16, or 80 per cent. of the control guinea-pigs, died.

A three weeks' old calf was injected over a period of five months, with remissions of one and two months, with a series of doses of toxin increasing from 4 mgm. to 800 mgm. Samples of serum tested during immunisation demonstrated increases in the mouse protective titre. The serum from the final bleeding was tested in mice against the intraperitoneal injection of culture. Doses of 0.4 ml. and 0.1 ml. respectively protected 20/20 and 18/20 mice against 0.1 ml. of culture, and 19/20 and 9/20 against 0.2 ml. of culture. In comparable groups of mice, injected with normal calf serum, the corresponding survivals were 4/20 and 5/20 against a challenging dose of 0.1 ml. and 1/20 and 0/20 against 0.2 ml. In control groups injected with 0.1 ml. and 0.2 ml. respectively of culture, 0/20 and 1/20 survived. The protective properties of the serum against the homologous strain was compared also with commercial *Bact. coli* anti-serum, using a single challenging dose of 0.1 ml. of culture. With three groups of ten mice in each estimation, the 50 per cent. protection end point of the endotoxin antiserum was 0.02 ml. and

of the control serum 0.643 ml. Serum protected animals observed for 42 days remained normal.

*Relative Toxicity of Bact. coli Endotoxin in Mice and Guinea-pigs and the Relationships between Dose and Mortality in the Two Animals.*

The relative susceptibility of mice and guinea-pigs to *Bact. coli* endotoxin was investigated by estimating the LD<sub>50</sub> of a number of batches of the isolated complex in both species. The data in Table V show that the lethal dose for guinea-pigs by subcutaneous injection was approximately equal to the lethal dose by intravenous injection in mice. This was in agreement with earlier observations that filtrates of heated cultures with a LD<sub>50</sub> for guinea-pigs of 1 ml. or 2 ml. were innocuous for mice in doses of 0.5 ml. On a weight basis, guinea-pigs were thus 17.5 times more susceptible than mice to *Bact. coli* endotoxin. This difference might have been still greater if comparable routes of injection had been employed.

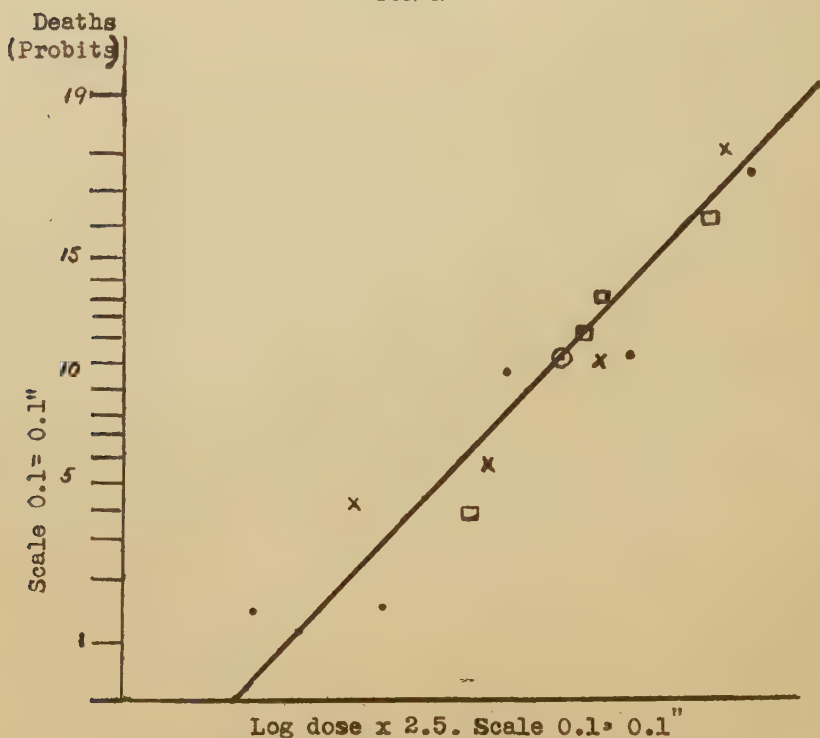
TABLE V  
RELATIVE TOXICITY OF PURIFIED CONCENTRATED *B. coli* ENDOTOXIN IN  
GUINEA-PIGS AND MICE

| Guinea-pigs subcutaneously |      |           |                  | Mice intravenously |           |                  |
|----------------------------|------|-----------|------------------|--------------------|-----------|------------------|
| Batch                      | Dose | Mortality | LD <sub>50</sub> | Dose               | Mortality | LD <sub>50</sub> |
|                            | mgm. |           | mgm.             | mgm.               |           | mgm.             |
| 56                         | 0.41 | 8/20      | 0.66             | 0.41               | 3/20      | 0.82             |
|                            | 0.82 | 10/20     |                  | 0.82               | 10/20     |                  |
| 57                         | 0.77 | 20/40     | 1.12             | 0.70               | 1/18      | 0.70             |
|                            | 1.55 | 11/20     |                  | 1.40               | 10/20     |                  |
|                            | 3.10 | 30/40     |                  | 2.80               | 19/20     |                  |
| 60                         | 0.52 | 10/20     | 0.52             | 0.26               | 2/20      | 0.50             |
|                            |      |           |                  | 0.65               | 15/20     |                  |
| 75                         | 0.43 | 7/40      | 0.81             | 0.29               | 2/10      | 0.58             |
|                            | 1.70 | 18/20     |                  | 0.58               | 10/10     |                  |
| 76                         | 0.76 | 12/20     | 0.57             | 0.38               | 2/10      | 0.76             |
|                            |      |           |                  | 0.76               | 5/10      |                  |
| 87                         | 0.52 | 4/20      | 1.14             | 1.30               | 4/20      | 1.80             |
|                            |      |           |                  | 2.60               | 17/20     |                  |
| 93                         | 1.36 | 9/20      | 1.47             | 0.78               | 1/10      | 1.40             |
|                            |      |           |                  | 1.55               | 6/10      |                  |
| 116                        | 0.24 | 5/20      | 0.58             | 0.48*              | 1/10      | 0.53             |
|                            | 0.60 | 11/20     |                  | 0.95*              | 6/10      |                  |
|                            | 1.20 | 14/20     |                  | 1.92*              | 10/10     |                  |

\* These mice were injected intraperitoneally, and the resulting LD<sub>50</sub> of 0.80 mgm. used to estimate the LD<sub>50</sub> which would result if the injections had been given intravenously.

The relation between dose of endotoxin and mortality in groups of 20 guinea-pigs is shown in Fig. 1. Fig. 2 represents the dose/mortality relation in mice and it is seen that, although the dosage in this figure is shown on a scale magnified to twice that of Fig 1, the slopes are approximately similar in the two figures. Thus, in order to produce a given increase in mortality, the dose would have to be increased twice as much in a guinea-pig experiment as in a

FIG. 1.

FIG. 1.—Dose/mortality relation of *Bact. coli* endotoxin in guinea-pigs.

- |                               |                                       |
|-------------------------------|---------------------------------------|
| ● Filtrate of heated culture. | □ Concentrated purified endotoxin.    |
| × Culture filtrate heated.    | ○ Calculated 50 per cent. end points. |

mouse experiment. Hence, if homogenous populations could be assumed in both species, this might be taken as an index of the greater accuracy of mouse tests in the measurement of endotoxin.

Toxicity in mice was about 50 per cent. greater by intravenous injection than by subcutaneous or intraperitoneal injection. In groups of 20 mice, 0.04, 0.02 and 0.01 ml. of a toxic solution killed 14, five and six mice by intraperitoneal injection and 15, 15 and three mice by intravenous injection. The same toxin, by subcutaneous injection in doses of 0.0107, 0.016, 0.024, 0.036, 0.054 and 0.081 ml. killed five, six, 12, nine, 16 and 18 mice. The calculated values of the

FIG. 2.

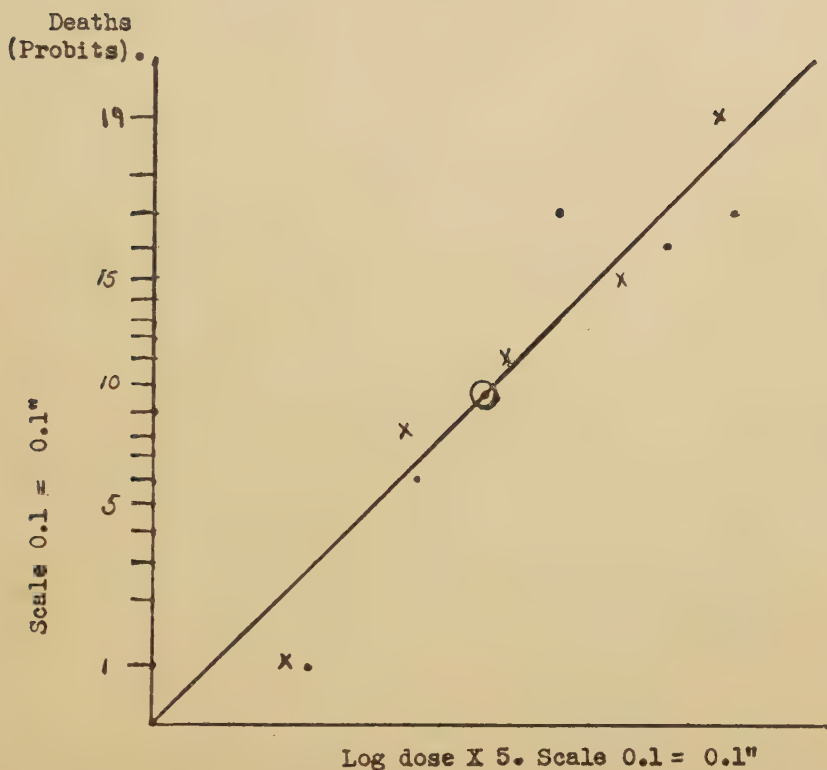


FIG. 2.—Dose/mortality relation of the endotoxins of *Bact. coli* and *Salm. enteridis* in mice.

● *Bact. coli* (60)

× *Salm. enteritidis* (6).

○ Calculated  $LD_{50}$  of each toxin.

The data for *Bact. coli* in this experiment are recognised to be inadequate for plotting, but they are supported by the results shown in Table V.

50 per cent. end points by the three routes were:—Intravenous, 0.017 ml.; intraperitoneal, 0.025 ml.; subcutaneous, 0.025 ml. The series of subcutaneous injections in this experiment also demonstrated that a steeper slope was not obtained by this route.

Statistically probable results were obtained throughout in guinea-pig experiments (Table I). The use of mice, however, was frequently attended with irregular results, examples of which may be seen in the preceding experiments. Discrepant results were sometimes obtained when toxicity was measured on separate occasions, and it was assumed as a working hypothesis that the value of  $LD_{50}$  for mice might fluctuate over an interval of  $\times 2$  in different experiments. In general, however, mortalities following intravenous injections were consistent with the slope in Fig 2, and there was, in the great majority of tests, no evidence of the presence of any

substantial proportion of highly susceptible or highly resistant mice. The data in Table V support this statement.

*Comparative Studies of the Endotoxins of Other Enteric Organisms*

The filtrate of an aerated culture of *S. enteritidis* in asparagine medium was innocuous to guinea-pigs in a dose of 8.0 ml. The filtrate of the same culture, heated at 80° C. for one hour, killed 5/20 guinea-pigs in a dose of 5 ml. In another experiment the filtrate of living culture killed 3/20 guinea-pigs in a dose of 10 ml., while the filtrate of the corresponding heat killed culture killed 12/20 in a dose of 4 ml. and 5/20 in a dose of 1 ml.; the centrifuged, unwashed organisms from the heat killed culture killed 6/20 guinea-pigs in a dose corresponding to 8 ml. of the culture. A saline suspension of *S. enteritidis* was standardised by opacity to 35,000 million organisms per ml., and heated at 80° C. for one hour; the filtrate of this killed 8/20 guinea-pigs in a dose of 0.2 ml. and 17/20 in a dose of 1 ml., a LD<sub>50</sub> of 0.31 ml. The organisms from the heated suspension were extracted with diethylene glycol and the extract, in a dose corresponding to 2.2 ml. of the original suspension, killed four out of 20 guinea-pigs, but gave no precipitate with alcohol. Thus, if the LD<sub>50</sub> of the extract is assessed at only 3.1 ml., the amount of endotoxin recovered from the heated cells would be only 10 per cent. of that found in the filtrate. A dose of 5 ml. of a filtrate of *Sh. shigae* culture killed 1/20 guinea-pigs, while the filtrate of the corresponding heat killed culture killed 9/20 in a dose of 5 ml. and 1/20 in a dose of 1 ml. In another experiment 5.0 ml. of a filtrate of living culture killed 0/20, while the same dose of the filtrate of heat killed culture killed 7/20. The organisms from the heat killed culture used in these two experiments were suspended to volume in saline solution and again heated at 80° C. for one hour. The filtrates of these suspensions were injected in doses of 5 ml. into groups of 20 guinea-pigs. No symptoms were seen and no deaths occurred.

The method employed for the concentration of *Bact. coli* endotoxin was applied successfully to one culture of *S. typhi-murium*, to seven cultures of *S. enteritidis*, and in a modified form to two cultures of *Sh. shigae* in peptone broth.

The toxicity for mice and guinea-pigs of endotoxin complex from cultures of *S. typhi-murium*, *S. enteritidis* and *Sh. shigae* are shown in Table VI; the *S. typhi-murium* endotoxin, prepared from a hydrolysate medium, the *S. enteritidis* batch 6, in hydrolysate medium, and batch 9, in ammonium sulphate medium, were comparable in their mode of preparation with the *Bact. coli* material and their LD<sub>50</sub> for mice were similar. The greater toxicity of *S. enteritidis* batch 2 may have been due to precipitation on storage, while the large LD<sub>50</sub> in batch 5 and in *Sh. shigae*, batch 98, were correlated with protein in the culture medium. The LD<sub>50</sub> of the *S. enteritidis* endotoxins was consistently less in mice than in guinea-pigs, the mean ratio being 1:3.66. The data suggest that guinea-pigs were

less uniform\* in their response to these endotoxins than to *Bact. coli* endotoxin. The degree of uniformity in the response of mice to *S. enteritidis* and *Bact. coli* endotoxin appeared to be identical.

TABLE VI  
TOXICITY OF THE PURIFIED CONCENTRATED ENDOTOXIN OF OTHER ENTERIC ORGANISMS IN GUINEA-PIGS AND MICE

| Endotoxin                           | Guinea-pigs subcutaneously |           |                  | Mice intravenously |           |                  |
|-------------------------------------|----------------------------|-----------|------------------|--------------------|-----------|------------------|
|                                     | Dose                       | Mortality | LD <sub>50</sub> | Dose               | Mortality | LD <sub>50</sub> |
| <i>S. typhi-murium</i> ...          | mgm.                       |           | mgm.             | mgm.               |           | mgm.             |
|                                     | 0.50                       | 7/20      | 1.50             | 0.50               | 2/10      | 0.72             |
|                                     | 1.00                       | 7/20      |                  | 0.75               | 5/10      |                  |
|                                     | 2.00                       | 11/20     |                  | 1.00               | 9/10      |                  |
|                                     | 4.00                       | 13/20     |                  |                    |           |                  |
| <i>S. enteritidis</i> , Batch 2 ... | 1.20                       | 11/20     | 1 approx.        | 0.17               | 1/10      | 0.23             |
|                                     |                            |           |                  | 0.35               | 8/10      |                  |
|                                     |                            |           |                  | 0.70               | 9/10      |                  |
| <i>S. enteritidis</i> , Batch 3 ... | ml.                        |           | ml.              | ml.                |           | ml.              |
|                                     | 0.30                       | 10/20     | 0.30             | 0.05               | 3/14      | 0.09             |
|                                     |                            |           |                  | 0.10               | 7/14      |                  |
|                                     |                            |           |                  | 0.20               | 14/14     |                  |
| <i>S. enteritidis</i> , Batch 5 ... | mgm.                       |           | mgm.             | mgm.               |           | mgm.             |
|                                     | 6.25                       | 9/20      | 7 approx.        | 1.25               | 4/20      | 1.62             |
|                                     |                            |           |                  | 2.5                | 20/20     |                  |
| <i>S. enteritidis</i> , Batch 6 ... | 0.60                       | 7/20      | 1.20             | 0.27               | 1/20      | 0.56             |
|                                     | 1.20                       | 9/20      |                  | 0.40               | 8/20      |                  |
|                                     | 2.40                       | 15/20     |                  | 0.60               | 11/20     |                  |
|                                     |                            |           |                  | 0.90               | 15/20     |                  |
|                                     |                            |           |                  | 1.34               | 20/20     |                  |
| <i>S. enteritidis</i> , Batch 7 ... | 0.25                       | 6/20      | 0.90             | No test.           |           |                  |
|                                     | 0.05                       | 9/20      |                  |                    |           |                  |
|                                     | 1.00                       | 7/20      |                  |                    |           |                  |
|                                     | 2.00                       | 13/20     |                  |                    |           |                  |
| <i>S. enteritidis</i> , Batch 9 ... | 0.50                       | 5/20      | 3 approx.        | 0.25               | 0/18      | 0.80             |
|                                     | 2.00                       | 8/20      |                  | 0.50               | 2/18      |                  |
|                                     |                            |           |                  | 1.00               | 12/18     |                  |
|                                     |                            |           |                  | 2.00               | 18/18     |                  |
| <i>Sh. shigae</i> , Batch 98 ...    | 3.90                       | 8/20      | 9.49             | No test.           |           |                  |
|                                     | 7.80                       | 8/20      |                  |                    |           |                  |
|                                     | 15.50                      | 13/20     |                  |                    |           |                  |
|                                     | 31.00                      | 16/20     |                  |                    |           |                  |

The *S. typhi-murium* complex contained 3.10 per cent. N and 6.94 per cent. carbohydrate in terms of glucose after hydrolysis; *S. enteritidis*, batch 6, contained 3.66 per cent. N and 14.3 per cent. of carbohydrate, while *Sh. shigae*, batch 98, contained 8.58 per cent.

\* In referring to animal experiments, the word "uniform" is used in its narrow sense to denote the regression of mortality upon dosage.

N and 13.10 per cent. carbohydrate. No attempt was made to pursue the study of chemical composition farther than was necessary to demonstrate that the material was a nitrogen containing compound which yielded sugar on acid hydrolysis. The final material obtained from *S. enteritidis* cultures sometimes precipitated on storage; it is not clear whether this phenomenon was a detachment of inert matter from the complex or reversion of the endotoxin complex into an insoluble form, nor is it possible to say whether it was more than fortuitous that it occurred with the *S. enteritidis* material. The phenomenon was noted in *S. typhi-murium* and *S. typhi* endotoxin complexes by Freeman *et al.* (1940).

The symptoms in guinea pigs did not appear to be different from those following *Bact. coli* endotoxin injections. In general post-mortem appearances were also similar, but the haemorrhagic splashes in the colon were seldom seen in guinea-pigs injected with *S. enteritidis* endotoxins and the intense congestive appearance of the small intestine appeared to be commoner.

#### DISCUSSION

It has been customary to regard the endotoxin of enteric bacteria as the substance isolated from the cell bodies by the relatively complex chemical methods of Boivin and Mesrobian (1933), Raistrick and Topley (1934) and Morgan (1937). It is important to note, however, that these toxic substances had been described in many earlier studies, such as those of Branham (1925), Ecker and Richardson (1925) and Steinberg and Ecker (1926) and that they had been isolated as polysaccharide complexes by Furth and Landsteiner (1929) and D. Smith (1927) by comparatively simple methods, regarded by the authors as alkaline extractions. Although at that time the endotoxin was looked upon as capsular substance, Furth and Landsteiner (*loc. cit.*) were interested in a method of preparing endotoxin by trypsin digestion of cells; this and much subsequent work appears to have been influenced by the hypothesis that endotoxin was the core of the cell and that a digestion or maceration of the cells was necessary in order to extract it. Some workers appear to have reverted recently to simpler methods, *e.g.*, Zahl and Hutner (1943) treated synthetic medium cultures with 2 per cent. of phenol and eliminated media constituents by dialysis, and Smolens *et al.* (1946) heated acidified suspensions at 56° C. for 20 hours. In the method of Furth and Landsteiner and of D. Smith (*loc. cit.*) heating was an important part of the procedure. The results obtained in this work have shown that heat at pH 7.0 alone releases endotoxin from the cell. A temperature of 80° C. was used for expediency to ensure sterilisation, but 60° C. was probably as effective and it is clear that in liquid cultures some endotoxin passed into the supernatant at the incubation temperature of 38° C. The rapid increase in the solubility of concentrated endotoxin as temperature rises from 25° C.

to 80° C. suggests that there might be a relation between this and the release of endotoxin from the cell under the influence of similar temperatures.

There is in this work a series of observations which, taken together, lead to the possibility of preparing endotoxin on a large scale. The use of the aeration technique eliminates the laborious technique of cultivation on surface media, the use of heat obviates any complex procedure for the release of endotoxin from the cells, while the absence of any large quantity of non-dialysable constituents in the culture medium simplifies the isolation of the endotoxin complex. It is thus possible without special apparatus other than air filters, aerators, a Sharples centrifuge and a small industrial still to employ several hundred litres of dense culture to prepare endotoxin. It is important, therefore, to consider the properties of the preparation obtained by this method. Experimental work demonstrated that the complex had full biological activity. It was antigenic, but its antigenicity must be viewed against the background of uncertainty which exists in the literature on this subject. Morgan and Partridge (1941) and more recently Tal and Olitski (1948), working with the endotoxin of *Sh. shigae*, demonstrated the association of antigenicity with protein. It appears to be difficult to apply their theories when the organisms are grown in synthetic media. Its chemical composition may well differ from that of a complex prepared from agar growths in which wide differences are commonly recorded. The trypsin digestion preparations of Cameron *et al.* (1940) with 4 per cent. N and 50 per cent. of reducing sugar on hydrolysis are typical examples, but Boivin (1946) mentions a preparation with 2.1 per cent., N, while Smolens *et al.* (1946) found an average of 6.4 per cent. N. Boivin and Mesrobian (1938) found 46 per cent. and 22 per cent. of reducing sugar in the complex from two strains of *S. typhi*. The figures recorded here are thus compatible with those in other reports. There is insufficient evidence in the literature to determine whether composition varies according to species, but it is certain that it varies with the method of preparation. In the process described here it would be expected that the absence of agar polysaccharide might lower the amount of reducing sugar obtained on hydrolysis, that when hydrolysate is used in cultivation some non-dialysable nitrogenous material present in the hydrolysate might increase the N in the complex and that heating, if carried out at 80° C., might affect the composition of the complex by denaturation of bacterial protein.

Some interest attaches to the technique of cultivation by aeration because of the observation that the vigour of the aeration was of paramount importance. It is difficult to believe that if 0.2 cubic foot of air per minute was ineffective and 0.75 cubic foot was effective, that the procedure was one of oxygen supply. It would appear more probable that the effect obtained was due to the immediate

local removal of some metabolite; the work of Johnstone (1942) suggests that this could be hydrogen.

Results of interest were also obtained in experiments designed to determine the essential nature of the pathological changes in guinea-pigs injected with *Bact. coli* endotoxin. Gross appearance of lesions suggested capillary haemorrhage, while microscopic examination failed to determine whether the lesion was an extravasation or a hypertension. When the trypan blue technique which Menkin (1938) employed to detect capillary permeability was applied, no evidence of permeability could be obtained. These results thus supported the view of Delaunay *et al.* (1947) that the lesion was a terminal hypertension.

In so far as these researches are concerned with the possibility of immunological control of white scour in calves, it appears possible from the results obtained in the protection of mice that the preparation of an anti-serum deserves further study. The results obtained are in agreement with those of Smith (1927), who was able to protect guinea-pigs against death from intraperitoneal injection of culture with an anti-serum prepared with *Bact. coli* culture. The prospects of employing endotoxin for the stimulation of active immunity in para-dysentery were discussed by Smolens *et al.* (1946) and their conclusions probably apply to *Bact. coli* immunity; they argue that as there is no immediate likelihood of developing a method of detoxifying endotoxin and as bacterial cells are as effective as the complex in stimulating immunity, there is no obvious advantage in using endotoxin instead of bacterial suspensions as antigens.

#### SUMMARY

When suspensions of *Bact. coli* were heated at 80° C. for one hour, the endotoxin entered the suspending fluid and could be recovered from the filtrate. No evidence could be obtained that any appreciable amount of endotoxin had been left on the cells. The same phenomenon could be demonstrated in suspensions of *S. enteritidis* and *Sh. shigae*. It was established that the *Bact. coli* endotoxin liberated in this way had the physical, chemical and biological properties which are associated with the endotoxins of salmonella and dysentery organisms.

These observations were made by employing guinea-pigs to detect the endotoxin. Experiments on purified endotoxin complex showed that, weight for weight with the guinea-pigs injected subcutaneously and the mice intravenously, guinea-pigs were 17.5 times more susceptible than mice. It is possible that guinea-pigs may be more susceptible to *Bact. coli* endotoxin and more uniform in their response to it than to the endotoxins of other enteric bacteria.

Mice were more uniform than guinea-pigs in their response to all the endotoxins and their superiority as experimental animals, when sufficient endotoxin is present, is not in doubt.

The technique used for the mass cultivation of bacteria is recorded and a method of preparing endotoxin, based on the results of the investigation, is described.

The results of pathological examination and of some biological experiments supported the view that the essential lesion in guinea-pigs was terminal hypertension in the circulation without permeability of capillaries.

The serum of a calf which had been injected with progressively increasing doses of *Bact. coli* endotoxin complex protected mice against death from intraperitoneal injections of living culture of the homologous strain.

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THE LATENT PHASE OF SHEEP SCAB: ITS NATURE AND  
RELATION TO THE ERADICATION OF THE DISEASE

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## INTRODUCTION

A RECURRENCE of Sheep-Scab in infected flocks, which have been strictly isolated after treatment and apparent cure, is an established phenomenon. It has evoked frequent comment in the literature and Stockman (1910) devoted a special publication to a catalogue of the histories of these occurrences.

Because of the regularity of the apparent cure in most cases, the satisfactory nature of established treatment was not questioned and the scab-mites were credited with prodigious powers of survival, amounting to many months or even years when protected in crevices in the walls or among the litter of sheep-folds. Investigation has shown, however, that the mites do not survive long when removed from the host and the period of 31 days established by Stockman (1912) and extended to 38 days by Babcock and Black (1933), represented the extreme to which only one individual survived. The mite's vitality falls rapidly and the power to reinfest sheep was found to have been lost after 13 days off the host. The eggs have even lower powers of survival and they had lost their ability to hatch within seven days. Proof of these data under practical conditions was secured by du Toit (1923), when it was found that heavily contaminated pens were safe after having been kept vacant for 17 days.

The complete resolution of the symptoms of the disease during the summer, without recourse to treatment, has also been observed. This is only an apparent recovery, however, for the approach of autumn often brings a relapse even though the sheep have been kept in strict isolation and all possibility of reinfection excluded. Familiarity with this phenomenon induced Stockman (1910) to postulate an undetected quiescent stage of the mite and to admit that there may be aspects of its life history which must await demonstration before attempts at eradication could be secured on a rational basis.

The explanation for these relapses has only gradually come to light. As long ago as 1877, Zürn reported the presence of scab-mites in the ears of sheep to be of common occurrence. Imes (1916), stated that the mites are sometimes found in the ears and infra-orbital fossae and that fresh outbreaks may be attributed to the survival and dissemination of mites from these sites following imperfect dressing. Henry (1917) found psoroptic otacariasis in 15 of a group of 32 emaciated sheep which showed no appearance of body scab. Miller (1925) was familiar with the presence of the mites in the ears

and recommended special attention to this site. Verney (1926), in a description of the disease in Basutoland, recorded the prevalence of mites in the ears, orbital fossae, inguinum and scrotum. He considered these sites to be the reservoirs from which mites are derived to re-establish scab after apparent recovery of the sheep and insisted on the hand-dressing of these sites in measures for eradication. Though long known as a clinical feature of the disease this spontaneous recovery and subsequent relapse was first studied by Downing (1936), who made observations on eleven sheep in which the disease became suppressed during the summer. In three of these sheep the mites became extinct and the sheep made a complete recovery; in the remainder, a few mites could be found in small non-progressive lesions on the body, and although no mention is made of mites in the ears, colonies of mites were observed in the infra-orbital fossae and inguinal folds of some sheep, where they remained throughout the warm months. On the approach of winter the mites deserted their hiding places and clinical scab developed in five of the eleven sheep. This phase of the disease, which he believed to be strictly a summer phenomenon, Downing called "latent or suppressed scab."

In view of the great practical importance of these observations from the point of view of eradication of the disease it was decided to carry out a series of investigations in an attempt to add something to the findings of Downing, with particular reference to the seasonal incidence and the flock incidence of the phenomenon.

#### PRELIMINARY OBSERVATIONS

In the first of these observations which were incidental to dipping trials designed to test the results of dipping during the latent phase, 78 Hampshire Down hogs were infested in mid-April by contact with eight grossly affected mountain sheep. Extensive body scab had developed on most sheep before they were shorn in early July. Apparent resolution followed shearing and symptoms of itching and irritation disappeared. Although the sheep appeared to be free from infection, careful examination showed that all except six downland and four mountain sheep carried colonies of mites in various sheltered sites in which they persisted throughout the summer.

The ears and infra-orbital fossae were the most commonly affected of these places, but mites were also found in the inguinal pouches, interdigital fossae, perineum, scrotum, vulvar furrows or in folds in the thickened body skin protected by small amounts of crust. Table I shows the incidence of colonisation of the survival sites.

Twenty of the sheep were left untreated and in all of these clinical scab was re-established following the dispersal of mites from the latent colonies, with the approach of the autumn and regrowth of the wool.

On ten sheep the disease died out without any colonisation of the latency sites.

Thirty-eight of the original group of sheep were reinfected by inoculation in March of the succeeding year. These sheep were

TABLE I

SHOWING THE PERSISTENCE OF INFECTION IN VARIOUS SITES IN THE LATENT PHASE OF THE DISEASE IN 78 HAMPSHIRE DOWN SHEEP AND EIGHT MOUNTAIN SHEEP, INCLUDING SEVEN MALE SHEEP, DURING SUMMER, 1946

| <i>Sites in which mites were found</i> |                                     |             |                                      |                                   |                         |                |                                      |                       |
|----------------------------------------|-------------------------------------|-------------|--------------------------------------|-----------------------------------|-------------------------|----------------|--------------------------------------|-----------------------|
|                                        | <i>General<br/>body<br/>surface</i> | <i>Ears</i> | <i>infra-<br/>orbital<br/>fossae</i> | <i>In-<br/>guinal<br/>pouches</i> | <i>Vulvar<br/>folds</i> | <i>Scrotum</i> | <i>Inter-<br/>digital<br/>fossae</i> | <i>Peri-<br/>neum</i> |
| Number of<br>sheep ...                 | 12                                  | 71          | 70                                   | 49                                | 30                      | 7              | 25                                   | 15                    |
| Percentage                             | 13.9                                | 82.5        | 81.3                                 | 56.9                              | 36.9                    | 100            | 29                                   | 17.4                  |

found to be more resistant to this second infestation, despite more exuberant exudation and encrustation and mites appeared to increase more slowly. Mites were first observed in the latency sites on 8.5.47 (in the ears) and in many sheep these sites were fully colonised by 25.5.47. Mites persisted in 36 of the sheep after shearing on 26.6.47 and were able to survive and to re-establish the clinical disease after the wool had regrown. The proportion of organs colonised differed from the previous year and is summarised in Table II.

TABLE II

SHOWING THE PERSISTENCE OF INFECTION IN VARIOUS SITES IN THE LATENT PHASE OF THE DISEASE IN 38 SHEEP, INCLUDING ONE MOUNTAIN SHEEP AND TWO MALE SHEEP, DURING SUMMER, 1947

| <i>Sites in which mites were found</i> |                                     |             |                                      |                                   |                         |                |                                      |                       |
|----------------------------------------|-------------------------------------|-------------|--------------------------------------|-----------------------------------|-------------------------|----------------|--------------------------------------|-----------------------|
|                                        | <i>General<br/>body<br/>surface</i> | <i>Ears</i> | <i>Infra-<br/>orbital<br/>fossae</i> | <i>In-<br/>guinal<br/>pouches</i> | <i>Vulvar<br/>folds</i> | <i>Scrotum</i> | <i>Inter-<br/>digital<br/>fossae</i> | <i>Peri-<br/>neum</i> |
| Number of<br>sheep                     | 14                                  | 9           | 10                                   | 1                                 | 1                       | 1              | 0                                    | 10                    |
| Percentage                             | 36.8                                | 23.9        | 26.3                                 | 3.6                               | 3.7                     | 50             | 0                                    | 26.3                  |

*Summary of Preliminary Observations.*—The large number of observations condensed in these tables appeared to confirm the seasonal nature of the latency phenomenon and to justify the conclusion that the approach of summer and shearing initiates a rapid regression of the lesions and in many instances an apparent recovery of the sheep. As a rule, mites invade one or more of the following sites: the ears, infraorbital fossae, inguinal pouches, interdigital fossae, scrotum, perineum, vulvar folds, the tail or deep folds in the skin and persist there till the autumn when they invade the body surface again and bring about a return of the symptoms of the disease.

As all of the inoculations had been made at one season of the year, however, it was decided to make further observations following the setting up of infection at other times of the year.

#### EFFECT OF THE SEASON OF INOCULATION ON THE COURSE OF THE DISEASE

##### *Procedure*

For this purpose sheep were inoculated in pairs in October, November, January, April, May and August, and the course of the disease closely observed. In all cases (with few exceptions subsequently mentioned) the inoculum consisted of ten ovigerous female mites which were transferred on a needle-point from an infected sheep to the skin in the region of the wither of the experimental sheep. To aid subsequent examination a knot was tied in the tip of the lock of wool covering the infected site. All of these sheep were isolated subsequent to inoculation.

It had been intended to make examinations of the sheep at weekly intervals, but wetting of the fleece by rain made inspection unsatisfactory and prevented adherence to an exact time-table. In early examinations, the sheep were strapped on a trestle-table of the kind described by Downing. The ledges of this table provided excellent security for the base of a Greenough-type microscope, having a special arm with a universal joint which made all parts of the body accessible for examination. Restraint was aided by covering the sheep's head with a cloth. This method, however, was found to be unsatisfactory, the respiratory movements carrying the field in and out of the focus of the microscope and this prolonged exposure to light, stimulating the mites to move into cover of the wool. Naked-eye examination was found to be much more practical; it was much quicker and sufficiently certain for detecting and identifying with reasonable assurance all stages of the mites. When certain identification was necessary the mites were removed on a needle-point to a microscope and afterwards returned to the site of origin with as little disturbance as possible.

##### *Histories of Sheep Inoculated at Different Seasons*

The histories of a few of these sheep is given below in illustration of the seasonal changes.

##### *Sheep Inoculated in November*

Sheep 1813 (Six-month-old Cross-bred Downland Female).

1.11.47.—Ten ovigerous female mites transferred to withers. The disease became quickly established.

25.11.47.—Continuous lesions on withers covering two hands' area.

4.12.47.—Lesions now extended from withers to mid-lumbar region. No mites in any of the latency sites.

11.12.47.—Lesions extended withers to rump and down both shoulders. No denudation. Mites were now present in both infraorbital fossae and left ear.

17.12.47.—Lesion extended to neck, rump and flank. Small patch denuded from both shoulders, crusts rather loose and resembling bran. Mites now very greatly increased in number (over 40) in both infraorbital fossae, both ears, both inguinal pouches and all interdigital fossae.

22.12.47.—Lamb was now losing condition rapidly; denudation of wool extended to one hands area, both shoulders.

28.12.47.—Lamb died.

### *Sheep Inoculated in January*

Sheep 1917 (Twelve-months-old Cross-bred Female)

22.1.48.—Inoculated.

27.1.48.—Colony established as one continuous lesion.

12.2.48.—Lesions now extended to area of two hands.

23.2.48.—Lesion greatly extended from mid-thoracic region to tuber coxae.

3.3.48.—Spread from withers to rump, down both shoulders and beginning to extend down to flanks. Mites confined to edge of lesion, none in any of the latency sites and no denudation of wool.

8.3.48.—Lesions extended to points of the elbow on each side, about a hand's area denuded from both shoulders. More than ten mites now present in each ear and about six in the right infraorbital fossae, none in interdigital fossae, inguinum or perineum.

23.3.48.—Lesions extended to upper portions of the limbs. Great denudation of wool from shoulders and rump, mites still abundant on body. Mites now present in both ears, both infraorbital fossae, but not in inguinum, which was very greasy, nor in interdigital fossae nor perineum.

15.4.48.—Body lesion greatly regressed, moist edges now dry and number of mites greatly reduced, body greatly denuded, bare from withers to rump and shoulders.

25.5.48.—Active lesion with mites now present only on the neck, all other body lesions now dry. Crusts lifting over withers and rump and new growth of wool begun.

14.7.48.—Body quite free of scab and crusts completely disappeared. Numbers of mites in ears now greatly reduced and wax accumulating. In each infraorbital fossa the crusts had reduced the amount of feeding area, available and mites numbered only three or four.

19.8.48.—Wool beginning to grow. Mites still present in both infraorbital fossae and ears. Active lesion with moist exudation and mites now started in perineum, both flanks and region of the tail.

### *Sheep Inoculated in April*

Sheep 1947 (Twelve-month-old Dorset Horn Female)

15.4.48.—Inoculated.

22.4.48.—Small lesion established, two mites present.

29.4.48.—Lesion dry, mites dispersed.

24.5.48.—Reinoculated.

14.7.48.—Diffuse, small, dry lesions, dispersed over the body with very few mites present. Wool risen and pulled out of flanks and rump. Little encrustation in infraorbital fossae, about five mites present in each. Both ears waxy and but a few mites confined to the diverticulum.

Inoculation during the early summer was followed by the migration of the mites to the latency sites even though the general body surface was not greatly infested.

*Sheep Inoculated in August*

Sheep 1907 and 1912 (Full-fleece Dorset Lambs)

4.4.48.—Inoculated.

8.8.48.—All mites dispersed from site of inoculation, none present in latency sites and none found elsewhere on the body. Inoculation repeated. Both had now received 100 ovigerous female mites.

19.8.48.—All mites dispersed from site of inoculation and no lesion found on body surface. No mites found in the ears all of which were waxy. Two mites were present in the left infraorbital fossa but the other fossa was vacant. In the other sheep of the pair 1907, one mite was present in the right fossa but none elsewhere.

These points from the history of the course of the infestation in sheep 1907 and 1912 show that the summer inoculation was followed in these two sheep by the disappearance from an unsuitable body surface, though the animals were carrying a full fleece. The very small proportion of mites which reached the fossae, compared with the total inoculum, suggests that there is no directed migration on the part of the mites, but rather a random dispersal in the course of which some few mites do find themselves in favourable sheltered habitats.

EFFECT OF SHEARING AND DENUDATION OF WOOL ON THE REGRESSION  
TO THE LATENT PHASE OF THE DISEASE

It can be seen from the above histories that colonisation of the hidden refuges was closely related to the onset of denudation. It was also seen during both the 1946 and 1947 seasons that shearing brought about a remarkable change in the mite population and in the appearance of the lesions. Examination only one day after shearing revealed a very great reduction in the number of mites; where they had previously been abundant only a few survivors could be found, although the dessicated remains of dead mites could be found on many parts of the body, suggesting that there had been a high rate of mortality.

The inflammation and exudation ceased abruptly and the lesions were quickly resolved; the scabs became dry and in a few days had begun to lift from the skin of the sheep. New wool soon began to grow, though often unlike the normal wool of the sheep in texture and pigmentation. In Down sheep the colour was sooty-brown or black, in Herdwicks grey-brown, and in the Welsh sheep a bright orange-tan similar to that of the face of many Welsh sheep.

The apparent importance of shearing in the change in the clinical picture was further investigated during the second season. Two mountain sheep were inoculated on 25.10.46 and were close-shorn on 8.1.47. At the time of shearing both sheep had a similar appearance; the lesions were in one continuous area extending from the withers to the mid-lumbar region; there was a little thick encrustation, though mites were abundant and confined to the margin of

the progressing lesion. There were no mites in any of the latency sites. Subsequent to shearing the mites completely disappeared from both sheep, none were observed to attempt colonisation of the latency sites and the lesions were quickly resolved. Attempts to re-establish the disease on these denuded sheep by the transference of mites were unsuccessful, showing the unsuitability of the denuded skin.

Six of the group of sheep, particulars of which are shown in Table II, already heavily infested and already supporting colonies of mites in the latency sites, were left in full fleece when the rest of the group were shorn on 26.6.47. On the shorn sheep, denudation brought a very great reduction in the numbers of mites on the body surface, though small numbers survived on heavily encrusted sheep. On the full-fleeced sheep the active disease continued unmodified and the lesions remained moist-edged and progressive. Colonies of mites were established in the refuges in both groups of sheep before shearing and no difference could be noted in the survival of the latent colonies in short- and full-fleece sheep.

The scab established on full-fleece sheep may continue in the active phase despite the onset of the warm, dry weather and the rising of the wool since the moist lesions may provide adequate local humidity. On the other hand, attempts to set up the disease even on full-fleece sheep generally failed during this period. It has been found, however, that if the sheep's fleece is saturated by rain or by spraying a few hours before inoculation and kept humid for a few days, active disease can invariably be established at any time, despite hot, dry climatic conditions. From these observations it may be deduced that the sudden exposure of the skin through shearing, while causing the dispersal of the mites from the active lesions, does not cause any directed migration to the latency sites and the colonisation of refuges must begin before shearing if the disease is to survive in the latent phase till wool is regrown.

Cameron (1925), Shilston (1915) and others have shown that mites are rapidly killed on exposure to sunlight or to dryness and the change in these climatic factors in shorn or denuded sheep appears to offer an adequate explanation for the virtual extermination of mites from exposed body lesions.

#### THE STAGES IN THE DEVELOPMENT OF THE LATENT DISEASE

The stages in the production of lesions during the active period were noted during the general observations on the establishment of mites on inoculated sheep. The mites were seen to begin feeding within a few hours of transference; after each mite had fed, a minute inflamed tumour developed, followed by vesication, rupture and exudation of serum. Generally, these vesicles were transformed into pustules which increased in size before rupture and discharge. A variation in this sequence of events was observed with increasing frequency as the summer approached; it was also seen in sheep having a short, open, dry fleece or on the skin of sheep which had

begun to dry, following extensive encrustation and denudation. On these sheep the minute reddened tumours occasionally returned to normal without vesication or, if vesication did take place, the vesicle did not rupture but became desiccated and detached from the skin as a minute scab. This difference in detail explains the disappearance of the ordinary clinical appearance of scab on the body during the period of short fleece or denuded skin, though a few mites may still be present at the time.

In the active disease, however, vesication and exudation is the rule with coagulation of serum and pus to form the characteristic extending, moist, yellow, greasy scab of the active lesion. Production of this scab forces the mites continually outward to intact skin so that an active lesion shows a progression of changes. On the margin of the extending lesion is a narrow zone of inflamed moist skin where most of the feeding sites coalesce. This inflamed band surrounds an area where the exudation becomes transformed into soft, moist crusts which become paler and harder as they dry out towards the centre, from which the activity had started. Mites are generally to be found in numbers only in the inflamed zone at the margin of the lesion, but in older cases they may also be found recolonising the skin underneath the dry crusts which have begun to rise with the growth of new wool. Eventually, the encrustation spreads over most of the body surface. In the last stages when the spread is most rapid the wool becomes loosened and is easily pulled out by the sheep. Meanwhile, the whole appearance of the lesion changes; the active, moist edge becomes indistinct and scaly, the mites decrease in numbers and, as suitable feeding sites are no longer available, they disperse at random over the body surface. In this dispersal some of their number may reach the latency sites and are able to establish themselves.

In the matter of this dispersal it can be seen, from the histories given above, that colonisation of the latency sites begins some 45 to 60 days after the onset of the active phase of the disease, when extensive encrustation and denudation has rendered the general body surface unsuitable. During the summer months, however, it can be seen that the mites may be forced to disperse before the climax of the disease, either through a change in the climate or physiology of the fleece or by a combination of both. It can also be seen from these histories that mites may be found in the ears and other latency sites at any time of the year. This is of great importance in the diagnosis and treatment of sheep scab.

#### *General Appearance of the Latent Disease*

Two aspects of the latent phase of the disease must be considered: first, the survival of colonies of mites in areas on the general woolly surface of the body, such as the perineum, scrotum, etc., where they are protected from direct sunlight and excessive dryness, and, second, the colonisation of wool-less pouches, such

as the ears, infraorbital fossae and inguinum. The reaction of these two classes of sites to colonisation by the mites is very different. In the perineum, scrotum and vulva the reaction is similar to that shown by the general body surface, the lesions being merely extensions of those on the woolly parts. In these places acute, moist inflammation occurs, with much encrustation, followed by loss of wool and thickening of the skin and it is in the thick crusts which are produced in these areas that mites survive. A similar sequence occurs on the tails of mountain sheep, but here the encrustation remains firmly attached and continues to accumulate, the skin of the tail becomes thicker and is thrown into deep folds, causing the tail to take on the appearance of a "bell-rope." Very different, however, is the reaction of the ears, infraorbital fossae, interdigital fossae and inguinal pouches. Examination of these hairless sites in the early stages of colonisation shows very little inflammation with its associated vesication, exudation and encrustation, while manipulation of these sites did not produce the characteristic nibbling or kicking reaction. This lack of reaction is particularly noticeable in the inguinum. Although much slower, however, the succession of events in these sites is similar to that seen on the general body surface. Crusts gradually make their appearance and, as the result of slow accumulation, may ultimately terminate the infestation. When the cavity becomes encrusted the mites are usually driven out and will perish if the general body surface is still unsuitable for re-establishment.

Despite encrustation, infestation of such sites as the ear must often escape detection, for post-mortem dissection sometimes revealed numbers of mites in the inner part of the aural tube, examination of which would have been impossible in the live sheep.

#### BIONOMICS OF THE MITES IN LATENCY SITES

Observations were made on the morphology and biology of the mites during the latent phase. The mites were disturbed as little as possible during the course of the observations, and full examination of the sites only took place on dead sheep, though it was often possible to distinguish instars and see eggs without disturbance, especially in the inguinum of live sheep.

#### *Morphology of the Mites in the Latent Sites*

In 1920 Henry described the mites taken from the ears of horses as a different species from those found in the lesions of body-mange. These mites were stated to be of larger size and to differ in the arrangement of the setae on the posterior lobes of the male. In the course of the present investigation a comparison was therefore made between mites removed from latency sites and those from the body lesions, but no such differences in size or structure could be observed. The degree of cover-slip pressure can account for varia-

tion in size and by displacement, the relation of structures and it is thought that the difference seen by Henry might perhaps be explained in that way.

### *The Communities in the Latent Sites*

The number of mites present, for example in the ears, varied greatly. In the summer they were generally few in number; in winter, on the other hand, they were often quite abundant. As many as 63 ovigerous females have been counted in one ear (9.8.46), in one case 73 females were counted in the infraorbital fossae, while in both inguinal pouches of one sheep more than 300 females were counted (1.11.47). Generally, the more severely a sheep had been affected during the active period, the greater the subsequent infestation in the latency sites.

A great preponderance of ovigerous females was seen in all latency colonies, and in many instances this instar alone was present. Typical proportions are exemplified in the following counts in Sheep 1531 which died 9.8.46:—

|                  | <i>Ovigerous<br/>females</i> | <i>Males</i> | <i>Pubescent<br/>females</i> | <i>Nymphs</i> | <i>Larvae</i> | <i>Eggs</i> |
|------------------|------------------------------|--------------|------------------------------|---------------|---------------|-------------|
| Left ear ...     | 63                           | 3            | 2                            | 2             | 7             | 17          |
| Right ear ...    | 43                           | 4            | 4                            | 5             | 9             | 11          |
| Left inf. fossa  | 73                           | 4            | 3                            | 2             | 8             | 11          |
| Right inf. fossa | 41                           | 3            | 1                            | 3             | 11            | 9           |

### *Oviposition*

Downing (1936) and others have shown that, in normal circumstances, females lay about two eggs per day, and these eggs take a little more than two days to hatch. In theory, therefore, females in full lay should each be accompanied by four or five unhatched eggs, and material taken from active lesions does, in fact, show a proportion of eggs to females approximately of this order.

Data, as exemplified above, show evidence of a greatly reduced rate of oviposition which holds for the ears, inguinum, infraorbital fossae and interdigital fossae at all times of the year; and in the perineum, scrotum and other parts when the lesions become dry and non-progressive. Solomon (1937) has shown with another species of mite that a decrease in the humidity greatly reduces the rate of oviposition and of other vital processes, and it seems likely that a similar reason may be advanced for this change in the rate of egg-laying in the scab mites.

### *Activity and persistence of the mites*

Sheep scab mites at a period of rapid egg production have been shown by Downing (1936) to live for shorter periods than those laying smaller numbers of eggs. In the present series of observa-

tions it was concluded that mites in the latency sites were able to survive for long periods. In one case a single female mite was observed to be present in the ear of one sheep from 10.7.46 to 17.9.46. It seems unlikely that this mite was superseded by her own progeny, as none of the few eggs which were laid were observed to hatch and it is thought unlikely that the mite was replaced by another individual. If this deduction is correct the duration of life was at least 69 days, which is considerably in excess of any previous record and probably reflects the reduced activity of mites in these sites.

The reduced pathological reaction in the latency sites doubtless refers in part to a greater tolerance of the skin. Where communities of mites were large, however, the inflammation and ultimate encrustation was frequently sufficient to crowd the mites out of the site altogether. The evidence obtained strongly suggests that the activity of the mites is also greatly reduced. The production of wax in the ears and of physiological skin secretion in the other sites appears to be responsible for some reduction in the numbers of mites. As complete recovery from severe body scab approaches, along with the improvement in general health, a resumption of wax secretion was observed in the ears and of skin secretion in the fossae. The mites were seen to become entangled in this material and to die, so that in several instances the reaction of the sheep was able to drive out or destroy all the mites from the latency sites before the woolly surface was suitable for re-establishment of body scab. Such sheep recovered completely. On the other hand, a type of almost permanent infestation of the ears can occur. In Sheep 1849 the ears were seen to become colonised by 24.10.47, and on 19.8.48 were still occupied without any change in the appearance, though the history of continuous scab without loss of fleece on this sheep suggests that the ear colonies may have been replenished with mites from the body surface.

#### *The Relation of Host to Parasite during the Latent Phase*

Psoroptes has been found on many ruminants and in some appears to be confined to the ears. In the goat it was first reported by Geddoelst (1909), subsequently by Roubaud and Saceghem (1916), and by many others; in this host it is strictly confined to the ears. It has also been reported from gazelle by Caparini (1887), from antelopes by Canestrini (1894), and from the Big-horn *Ovis montana*, a hairy wild sheep of America, by Ward (1915). In the Equidae, Henry (1920) recorded the parasite as very prevalent in the ears of horses, asses and mules in France; in some depots 70 per cent. of the animals were affected without showing symptoms of body lesions.

It seems probable, therefore, that in wild ruminants and solipeds Psoroptes is restricted to the ears, where it is well tolerated and may be regarded as a normal, relatively benign ear-parasite. In short-haired animals, as in the goat and in native hairy sheep of Africa,

the body disease is unknown, and in cattle and horses the body disease occurs only in the winter when the coat is long or the animal housed or rugged. It seemed likely, therefore, that an equable micro-climate and refuge from light are the important points to allow colonisation of the body and that the close fleece of domesticated sheep very adequately fulfils these requirements. The denuded body, following gross body disease or the approach of summer which permits the drying of the lesions and the access of light, deprives the mites of this extended habitat and forces a retreat to the primitive foci, so that the shorn or denuded body of the sheep approximates to that of the primitive sheep in which *Psoroptes* and its host have evolved a balanced relationship. It is interesting to note that this retreat may begin before denudation occurs and may be in the nature of a premonitory migration. There is no evidence, however, for a purposeful migration to the primitive sites which are occupied during the latent period of the disease in sheep, but only of random dispersal when the general body surface becomes untenable.

#### *Relationship of the Observations to Control of the Disease*

While the year-round presence of mites in the hidden refuges has been established and is of obvious importance in diagnosis and eradication, it presents no difficulties in the cold season of the year. At that time of the year mites are present in the latent sites only as a sequel to extensive invasion and subsequent encrustation of the general skin of the body, so that only sheep which show signs of having been grossly infested need be suspected of carrying mites in the ears and other latency sites. The difficulties are greater during the summer months, as recently infected sheep may then have completed the recovery of small body lesions before shearing-time and mites dispersed from these unnoticed lesions may be present in the latent site quite unsuspected. With regard to treatment, however, presence of mites in the hidden refuges is of great importance whatever the time of the year on account of their inaccessibility to the dipping fluid.

Stockman (1910) described how relapses took place in 16 flocks of sheep which had been treated by dipping, kept in strict isolation and had apparently been cured. The present observations showing that mites may inhabit the latency sites at any season of the year appear to offer a satisfactory explanation for these relapses. The very long survival of mites in the ears, for example as in sheep 1849, suggests that hand treatment would be necessary to ensure eradication and that an acaricide of extraordinary persistence would be required to protect the body against reinfestation from the ears.

#### SUMMARY

The latent clinical phase of sheep-scab, in which symptoms could no longer be detected and the sheep regained their normal appear-

ance, despite the continued presence of hidden colonies of mites, was studied in 134 sheep.

Of 86 sheep, which had been experimentally infected with scab, all showed disappearance of symptoms after shearing, but in no less than 76 of them, mites could still be found in the latency sites. Twenty of these apparently healthy sheep were kept untreated and in every instance a recrudescence of clinical scab occurred when the wool was regrown. A repetition of these observations on 38 sheep showed a similar appearance with recrudescence of clinical scab in all but two of their number.

A similar regression to latent disease occurred during the winter months in six sheep, some six to ten weeks after inoculation, following the active phase of the disease which had produced much encrustation and denudation. In four other sheep infected during the summer months mites invaded the latency sites direct from the site of inoculation without first establishing themselves on the body surface.

#### CONCLUSIONS

In sheep scab there are two forms of latent disease. The first is seasonal and follows climatic and physiological changes which make the fleece and skin unsuitable for the mites. These changes are brought about by the approach of summer. The second occurs in the normal course of the disease irrespective of season; it is brought about by the extension of encrustation, the hardening and drying of the skin and the denudation of wool which produce conditions unsuitable for the mites.

In both forms the unfavourable conditions cause a dispersal of the mites from the lesions on the general body surface which may become completely free from infection. Shearing or denudation generally completes the eradication of the mites from the body and the sheep may then regain a completely normal appearance.

The dispersal of mites appears to be a random scattering and not a directed migration. In the course of this dispersal some of the mites reach the sheltered sites that are associated with the latent form of the disease and set up colonies which may persist for some time. These hidden colonies may be found at any time of the year, but the latency is most complete in the summer months following shearing.

The order of frequency in which these latency sites are colonised is as follows:—ears, infraorbital fossae, perineum, scrotum, inguinal pouches, tail, vulvar region and interdigital fossae. As many as 82.5 per cent. of the sheep were affected in the ears during the summer of 1946.

The mites are well-tolerated in many of the hidden sites and may provoke only a very slight reaction. In some instances, however, the mites may be destroyed by accumulation of wax and other secretion, or be driven out by the extension of crusts; although, as

a rule, they survive to re-establish the disease by recolonisation of the body surface when the wool has been regrown.

Examination of these latency sites is of great importance in establishing diagnosis. The inaccessibility of these latent colonies, especially in the ears, seems to explain many of the relapses after apparently effective treatment by dipping and indicates the importance of local treatment.

#### ACKNOWLEDGMENT

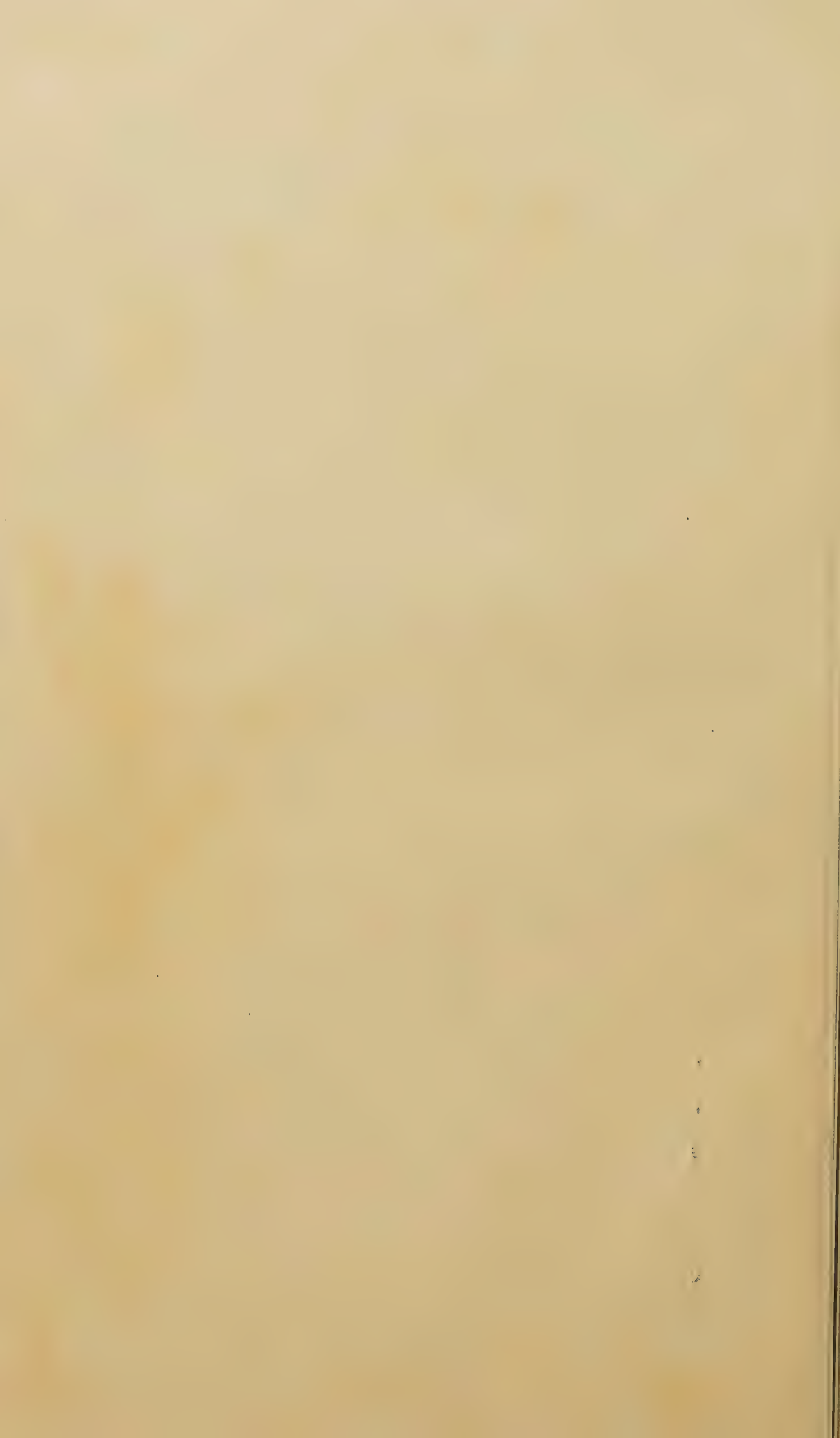
I wish to express my gratitude to Dr. E. L. Taylor for his help in the preparation of this paper.

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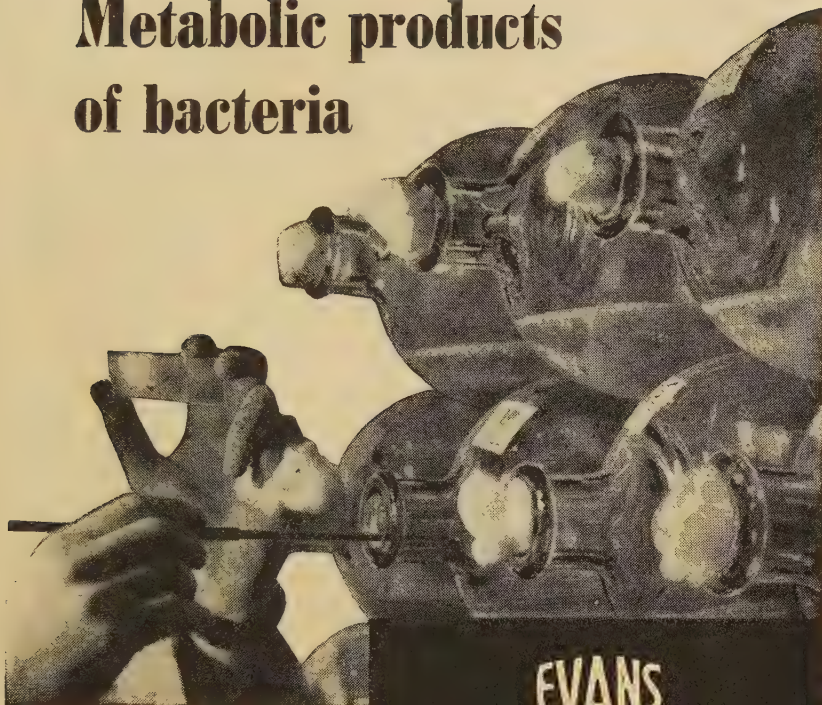
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